

WEST Search History

DATE: Friday, May 24, 2002

Set Name Query

side by side

Hit Count Set Name

result set

DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ

L22	l11 and l12 and ((calibrat\$ or standard) same curve?)	7	L22
L21	L20 and (calibrat\$ or curve?)	6	L21
L20	l16 and (control? or standard? or known? or reference?)	23	L20
L19	L18 and l16	2	L19
L18	L15 and ((calibrat\$ or standard) same curve?)	7	L18
L17	L16 and ((calibrat\$ or standard) same curve?)	2	L17
L16	L15 and (elisa or (immunosorbent assay?))	23	L16
L15	l1 and l11 and l12	36	L15
L14	l1 and (l11 same l12)	13	L14
L13	l1 same l11 same l12	2	L13
L12	cell\$4 same protein\$ same ((imaging) or (image analy\$5))	1136	L12
L11	cell\$4 same protein\$ same ((optical densit\$3) or (od))	1404	L11
L10	L9 and l8 and l6	2	L10
L9	((calibrat\$ or standard) same curve?) and l4	7	L9
L8	(elisa or (immunosorbent assay?)) and l4	23	L8
L7	(elisa or (immunosorbent assay?)) and l4	23	L7
L6	L5 and l4	23	L6
L5	(her\$3 or estrogen or prostate or psa or egfr or akt or p13 or map) same protein? same cell\$4	5428	L5
L4	L3 and (cancer or malignan\$3 or tumor\$ or tumour\$ or metasta\$5)	35	L4
L3	L2 and l1	36	L3
L2	cell\$4 same protein\$ same (((optical densit\$3) or (od)) and ((imaging) or (image analy\$5)))	55	L2
L1	cell\$4 same protein\$ same (assay? or immunoassay?)	12228	L1

END OF SEARCH HISTORY

WEST Search History

DATE: Friday, May 24, 2002

Set Name Query

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Hit Count Set Name

result set

DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ

L22	l11 and l12 and ((calibrat\$ or standard) same curve?)	7	L22
L21	L20 and (calibrat\$ or curve?)	6	L21
L20	l16 and (control? or standard? or known? or reference?)	23	L20
L19	L18 and l16	2	L19
L18	L15 and ((calibrat\$ or standard) same curve?)	7	L18
L17	L16 and ((calibrat\$ or standard) same curve?)	2	L17
L16	L15 and (elisa or (immunosorbent assay?))	23	L16
L15	l1 and l11 and l12	36	L15
L14	l1 and (l11 same l12)	13	L14
L13	l1 same l11 same l12	2	L13
L12	cell\$4 same protein\$ same ((imaging) or (image analy\$5))	1136	L12
L11	cell\$4 same protein\$ same ((optical densit\$3) or (od))	1404	L11
L10	L9 and l8 and l6	2	L10
L9	((calibrat\$ or standard) same curve?) and l4	7	L9
L8	(elisa or (immunosorbent assay?)) and l4	23	L8
L7	(elisa or (immunosorbent assay?)) and l4	23	L7
L6	L5 and l4	23	L6
L5	(her\$3 or estrogen or prostate or psa or egfr or akt or p13 or map) same protein? same cell\$4	5428	L5
L4	L3 and (cancer or malignan\$3 or tumor\$ or tumour\$ or metasta\$5)	35	L4
L3	L2 and l1	36	L3
L2	cell\$4 same protein\$ same (((optical densit\$3) or (od)) and ((imaging) or (image analy\$5)))	55	L2
L1	cell\$4 same protein\$ same (assay? or immunoassay?)	12228	L1

END OF SEARCH HISTORY

WEST



Generate Collection

L19: Entry 2 of 2

File: USPT

Jun 26, 2001

DOCUMENT-IDENTIFIER: US 6251586 B1

TITLE: Epithelial protein and DNA thereof for use in early cancer detection

Brief Summary Paragraph Right (13):

Another method for diagnosing human preneoplastic and neoplastic cells and tissues is by detecting post-translational modifications of the epithelial protein in the preneoplastic and neoplastic cells and tissue by immunoassays such as Western blot or immunoelectrophoresis using an antibody that is reactive with the epithelial protein, by two-dimensional electrophoresis or by reverse-phase HPLC.

Detailed Description Paragraph Right (21):

The protein, peptides and variants thereof may be used in diagnostic methods and in in vitro assays to detect the presence of a similar protein, peptide and variants thereof present in a biological sample. The assays allow for early detection of pre-neoplastic and neoplastic cells and in defining the process of carcinogenesis.

Detailed Description Paragraph Right (22):

In one embodiment, the isolated and purified protein, peptide or variant thereof is useful in immunoassays for the detection of the corresponding protein or variant thereof. The immunoassays are qualitative and quantitative. The immunoassays are useful in detection of precancer and cancer cells in which an increase in the quantity of the epithelial protein, peptide or variant thereof is indicative of precancer and cancer. Conversely, the immunoassays are useful in monitoring the efficacy of cancer treatment or intervention in which the absence or decrease in the quantity of the epithelial protein, peptide or variant thereof recovered from a patient undergoing treatment or intervention is an indication of an efficacious treatment.

Detailed Description Paragraph Right (23):

Immunoassays of the present invention may be a radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, chemiluminescent assay, immunohistochemical assay and the like and may be performed in vitro, in vivo or in situ. The standard techniques known in the art for ELISA are described in "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W. A. Benjamin, Inc., 1964; and Oellerich, M. 1984, J. Clin. Chem. Clin. Biochem., 22:895-904. Biological samples appropriate for such detection assays include, but are not limited to, cells, tissue biopsy extracts, whole blood, plasma, serum, sputum, cerebrospinal fluid, pleural fluid, urine and the like.

Detailed Description Paragraph Right (27):

The protein, peptide and variants thereof may be used to elicit specific antibodies and antigen binding fragments thereof that are immunoreactive with the epithelial protein, peptide or variant thereof. Of particular importance are antibodies or antigen binding fragment thereof that recognize an epitope which is associated with transformation of a normal cell to a pre-cancer cell. The epitope is not present or is present in low amounts in normal cells and is highly expressed in precancer and cancer cells. In one embodiment the antibody or antigen binding fragment thereof reacts with an epithelial protein, peptide or variant thereof having a post-translational modification, wherein said post-translational modification is indicative of a precancer or cancer cell. The antibodies may be produced by methods disclosed in U.S. Pat. No. 4,569,788 or by other methods known in the art. Such antibodies are useful in immunoassays to detect the epithelial protein and to detect post-translational modifications of the protein. The antibodies or antigen binding fragment thereof are useful as intermediate end-point markers in determining the

efficacy of a cancer treatment or intervention.

Detailed Description Paragraph Right (149):

The method relies on measurement of cellular features or labels whose expression differs as compared to typical or normal cells. These features or labels may include one or more of those listed below. Image analysis in combination with appropriate statistical software allows for the identity of cellular features which are predictive of the development of cancer. The image analysis detects differences or alterations in cellular features or labels distinctive of cancer and precancer. Various parameters may be labeled and measured as indicators or predictors of cancer including but not limited to alterations in morphology increased or altered mRNA expression, increased or altered cancer proteins, expression of a cellular receptor or alternatively a decline in cellular receptor, factors associated with apoptosis or other cellular events which are unique to precancer or cancer cells.

Detailed Description Paragraph Right (173):

The immunocytochemistry assay of sputum specimens had demonstrated a low level of background expression in normal sputum cells (U.S. Pat. No. 5,455,159), and provided the impetus to develop a dual-wavelength image densitometry technique to quantify enhanced antigen presence for early lung cancer detection (Tockman, et al. 1993, Diagnostic Cytopathol, vol. 9(6):615-22). Dual-wavelength image densitometry depends upon a series of carefully standardized and calibrated procedures (See FIG. 14) to assure reliable measurement of cytoplasmic optical density at 600 nm and 510 nm. Computerized interpretation of protein antigen densitometry combines these optical densities into a discriminant function.

Detailed Description Paragraph Right (180):

In FIGS. 16a-d, the expression of hnRNP A2 messenger RNA and protein is contrasted between a positive case (FIGS. 16a, 16b) and a negative case (FIGS. 16c, 16d). In the upper row, two aliquots of a specimen from a patient who later developed squamous lung cancer illustrates mild morphologic atypia and positive expression of hnRNP protein (FIG. 16a) and hnRNP messenger RNA (FIG. 16b). In the lower row, similar assays of the sputum of a patient who did not develop lung cancer show neither over-expression of protein nor of messenger RNA despite similar cellular morphology.

Detailed Description Paragraph Right (185):

For comparison, hnRNP protein expression in the same 13 sputum specimens is evaluated in a similar discriminant function analysis (Tables 20-24). Table 20 shows the group means and standard deviations for the optical densities measured on the immunostained sputum cells of individuals who later developed cancer and those who remained cancer-free. Although a trend is apparent, measurement variability and the small sample size preclude a significant difference (Table 21).

Detailed Description Paragraph Type 1 (8):

e. Plot density calibration. After averaging the transmitted light recorded by the CCD for dark, white, 1st and 2nd neutral density images the computer constructs a four point calibration curve of gray scale light intensity (on an 8-bit, 256 interval ordinate) against optical density (abscissa). One calibration curve is constructed for each wavelength. The calibration curves for the first day are arbitrarily selected as the standard curves, and calibration curves from the subsequent measurement sessions are standardized to these, assuring comparability of measurement values during the course of an experiment.

WEST



Generate Collection

L21: Entry 5 of 6

File: USPT

Sep 22, 1998

DOCUMENT-IDENTIFIER: US 5811098 A

TITLE: Antibodies to HER4, human receptor tyrosine kinase

Drawing Description Paragraph Right (20):

FIGS. 19A-D. Purification of p45 from HepG2 conditioned media. Column fractions were tested for their potential to induce differentiation of MDA-MB-453 cells. Active fractions were pooled as indicated by an horizontal bar. FIG. 19A, Concentrated HepG2 conditioned medium was subjected to 50% ammonium sulfate precipitation. Supernatant resulting from this step was subjected to hydrophobic interaction chromatography using phenyl-Sepharose. Pooled fractions were then loaded on a DEAE-Sepharose column. FIG. 19B, the DEAE-Sepharose column flow-through was subjected to CM-Sepharose chromatography. FIG. 19C, Affinity Chromatography of the MDA-MB-453 differentiation factor using heparin-5PW column. Fractions 35-38 eluting around 1.3M NaCl were pooled. FIG. 19D, Size Exclusion chromatography of the differentiation factor. The molecular masses of calibration standards are indicated in kilodaltons.

Drawing Description Paragraph Right (21):

FIG. 20. Aliquots (25 microliter) of the active size exclusion column fractions (30 and 32) were electrophoresed under reducing conditions on a 12.5% polyacrylamide gel. The gel was silver-stained. Molecular masses of Bio-Rad silver stain standards are indicated in kilodaltons.

Drawing Description Paragraph Right (23):

FIG. 22A-B. Binding and cross-linking of .sup.125 I-p45 to CHO-KI, CHO-HER2 and CHO/HER4 cells. FIG. 22A, Scatchard analysis of the binding of .sup.125 I-p45 to CHO/HER4 cells. Increasing concentrations of .sup.125 I-p45 were incubated with cell monolayers for 2 h at 4.degree. C. Nonspecific binding was subtracted from all cell-associated radioactivity data values. A Scatchard plot as well as a saturation curve of the binding data are shown. FIG. 22B, Covalent cross-linking. .sup.125 I-p45 was added to the cells in the presence or absence of an excess of unlabeled p45 for 2 h at 4.degree. C. After washing of the cells to remove unbound iodinated material, the cross-linking reagent bis-(sulfosuccinimidyl)-suberate was added to the cells for 45 min. at 4.degree. C. Cells were lysed and proteins separated by electrophoresis on a 7.5% polyacrylamide gel. Molecular masses of protein standards are indicated in kilodaltons. A Molecular Dynamics Phosphorimager was used to visualize the radioactive species.

Drawing Description Paragraph Right (26):

FIG. 25. Purification of the chimeric HAR-TX .beta.2 protein: shown is a Coomassie brilliant blue stained SDS-PAGE (4-20%) of the different purification steps. Lanes 1-5 have been loaded under reducing conditions. Lane 1, MW standards; lane 2, refolded HAR-TX .beta.2, 20.times. concentrated; lane 3, POROS HS flow-through, 20.times. concentrated; lane 4, POROS HS eluate; lane 5, Source 15S eluate (pure HAR-TX .beta.2, 2 .mu.g); lane 6, 2 .mu.g HAR-TX .beta.2, loaded under non-reducing conditions.

Drawing Description Paragraph Right (27):

FIG. 26. Membrane-based ELISA binding analysis, performed to determine the binding activity of the purified HAR-TX .beta.2 protein. Binding of HAR-TX .beta.2 (.smallcircle.) and PE40 (.circle-solid.) to membranes prepared from the HER4 expressing human breast carcinoma cell line.

Detailed Description Paragraph Right (101):

On further purification, the HepG2-derived factor was found to elute from a phenyl

hydrophobic interaction column at 1.0M ammonium sulfate (fractions 16 to 18). FIG. 11, Panel 4, shows the phenyl column elution profile. Tyrosine phosphorylation assays of the phenyl column fractions revealed that the same fractions found to induce differentiation of the human breast carcinoma cells are also able to stimulate tyrosine phosphorylation of a 185 kDa protein in MDA-MB-453 cells (FIG. 11, Panel 5). In particular, fraction 16 induced a 4.5-fold increase in the phosphorylation signal compared to the baseline signal observed in unstimulated cells, as determined by densitometry analysis (FIG. 11, Panel 6).

Detailed Description Paragraph Right (114):

cNHER2 and cNHER4 expression plasmids were generated by insertion of the complete coding sequences of human HER2 and HER4 into cNEO, an expression vector that contains an SV2-NEO expression unit inserted at a unique BamHI site of CDM8. These constructs were linearized and transfected into CEM cells by electroporation with a Bio-Rad Gene Pulser apparatus essentially as previously described (Wen et al., supra). Stable clones were selected in RPMI/10% FBS supplemented with 500 $\mu\text{g/ml}$ active Geneticin. HER2 immunoprecipitations were as described in FIG. 15, using 5.times.10.sup.6 cells per reaction, and the HER2 Western blots were performed with a second anti-HER2 Mab (c-neu Ab-3, Oncogene Sciences). For metabolic labeling of HER4, 5.times.10.sup.6 cells were incubated for 4-6 h in methionine and cysteine-free Minimal Essential Medium (MEM) supplemented with 2% FBS and 250 $\mu\text{Ci/ml}$ [³⁵S]Express protein labeling mix (New England Nuclear). Cells were washed twice in RPMI and solubilized as above. Lysates were then incubated for 6 h, 4.degree. C. with 3 μl each of two rabbit antisera raised against synthetic peptides corresponding to two regions of the cytoplasmic domain of human HER4 (.sup.864 LARLLEGDEKEYNADGG.sup.88 [SEQ ID No:31] and .sup.1010 EEDLEDMMDAEEY.sup.1022 [SEQ ID No:32]). Immune complexes were precipitated with 5 μg goat anti-rabbit Ig (Cappel) and Protein G Sepharose (Pharmacia). Proteins were resolved on 7% SDS-polyacrylamide gels and exposed on the phosphorimager. For Mab-stimulation assays, 5.times.10.sup.6 cells were resuspended in 100 μl RPMI and 25 $\mu\text{g/ml}$ Mab was added for 15 min at room temperature. Control Mab 18.4 is a murine IgG.sub.1 specific to human amphiregulin (Plowman et al., 1990, Mol. Cell. Biol. 10:1969-1981). Following Mab-treatment, cells were washed in PBS, solubilized (Section 13, infra), and immunoprecipitated with anti-HER2 Mab (Ab-2). Tyrosine phosphorylated HER2 was detected by PY20 Western blot as in FIG. 15.

Detailed Description Paragraph Right (143):

The plasmid pSE 8.4 encoding the chimeric protein HAR-TX .beta.2 was transformed into the E. coli strain BL21 (.lambda.DE3). Cells were grown by fermentation in T broth containing 100 $\mu\text{g/ml}$ ampicillin at 37.degree. C. to a optical density of A.sub.650 =4.8, followed by induction of protein expression with 1 mM isopropyl-1-thio-.beta.-D-galactopyranoside (IPTG). After 90 minutes the cells were harvested by centrifugation. The cell pellet was frozen at -70.degree. C., then thawed and resuspended at 4.degree. C. in solubilization buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin-A, 0.5 mM PMSF) containing 1% tergitol by homogenization and sonication. The insoluble material of the suspension, containing inclusion bodies with the HAR-TX .beta.2 protein, was pelleted by centrifugation and washed three times with solubilization buffer containing 0.5% tergitol (first wash), 1M NaCl (second wash), and buffer alone (third wash).

Detailed Description Paragraph Right (148):

HER4, expressed in baculovirus, was used as the immunogen for subcutaneous injection into 4-6 week old female BALB/c mice. Immunization was performed 4 times (approximately 1 month apart) with 20 μg of HER4 protein given each time. Spleen cells from immunized mice were removed four days after the final immunization and fused with the mouse myeloma line P2x63-Ag8.653 as previously described (Siegall et al., supra). Positive hybridoma supernatants were selected by ELISA screening on plates coated with HER4 transfected CHO cells (Plowman et al., 1993, Nature 366:473-475) and selected against parental CHO cells and human fibroblasts. Secondary screening was performed by ELISA on plates coated with baculovirus/HER4 membranes. Positive hybridomas were rescreened by two additional rounds of ELISA using CHO/HER4 and HER4 negative cells, and identified false positive were removed. Positive hybridomas were cloned in soft agar and tested for reactivity with the HER4 positives MDA-MB-453 human breast carcinoma cell line and CEM cells co-transfected

with HER4 and HER2. Anti-HER4 hybridoma line 6-4-11 (IgG1) was cloned in soft agar and screened for reactivity to native and denatured HER4. A second antibody (7-142, IgG2a) was also selected and found to bind to the cytoplasmic domain of HER4.

Detailed Description Paragraph Right (150):

Cell-surface expression of HER2, HER3, and HER4 protein was determined by quantification of specific antibody binding, detected by the CAS Red Chromagen system (Becton Dickson Cellular Imaging System, Elmhurst, Ill.). HER2 staining was performed by using mouse anti-HER2 mAb 24.7 (Stancovski et al., 1991, Proc. Natl. Acad. Sci. USA 88:8691-8695) as primary, and biotinylated goat anti-mouse IgG (Jackson Labs, West Grove, Pa.) as secondary antibody as previously described (Bacus et al., 1993, Cancer Res. 53:5251-5261). For detection of HER3 and HER4 the primary antibodies used were, respectively, mouse anti-HER3 mAb RTJ2 (Santa Cruz Biotech, Santa Cruz, Calif.) at 2.5 .mu.g/ml concentration or mouse anti-HER4 mAb 6-4-11 at 15 .mu.g/ml concentration followed by incubation with biotinylated rabbit anti-mouse IgG (Zymed Labs, South San Francisco, Calif.).

Detailed Description Paragraph Right (152):

Image analysis was performed as previously described (Bacus et al., 1993, supra; Bacus et al., 1992, Cancer Res. 52:2580-2589; Peles et al., 1992, Cell 69:205-216). In the quantitation of HER2, both solid state imaging channels of the CAS 200 Image Analyzer (Becton Dickinson Cellular Imaging System), a microscope-based, two-color system were used. The two imaging channels were specifically matched to the two components of the stains used. One channel was used for quantitating the total DNA of the cells in the field following Feulgen staining as described (Bacus et al., 1990, Mol. Carcinog. 3:350-362), and the other for quantitating the level of HER2, HER3, and HER4 proteins following immunostaining. When the total DNA amount per cell was known, the average total HER2, HER3, and HER4 per cell were computed. Sparsely growing AU565 cells were used for calibrating the HER2 protein. Their level of staining was defined as 100% of HER2 protein content (1.0 relative amounts=10,000 sum of optical density); all other measurements of HER2, HER3, and HER4 protein were related to this value.

Detailed Description Paragraph Right (156):

To determine the specific binding activity of HAR-TX .beta.2, an ELISA assay was performed using membranes of the HER4 positive human breast carcinoma cell line MDA-MB-453 as the target for binding. HAR-TX .beta.2 was found to bind to the immobilized cell membranes in a dose-dependent fashion up to 300 .mu.g/ml (FIG. 26). PE40, the toxin component of HAR-TX .beta.2 used as negative control, was unable to bind to MDA-MB-453 membranes.

Detailed Description Paragraph Center (86):

14.1.4. ELISA Test for Determination of Binding Activity

FILE 'SCISEARCH, BIOSIS, MEDLINE, USPATFULL' ENTERED AT 15:18:38 ON 24
MAY 2002

L1 196433 S CELL? (6P) PROTEIN? (6P) ?ASSAY?
L2 9402 S CELL? (6P) PROTEIN? (6P) ((OPTIC? DENSIT?) OR OD)
L3 13665 S CELL? (6P) PROTEIN? (6P) ((IMAGING) OR (IMAGE ANALY?))
L4 235230 S CELL? (6P) PROTEIN? (6P) (CANCER? OR MALIGNANT OR METASTA? O
L5 27914 S L1 (6P) (ELISA OR IMMUNOSORBENT)
L6 27914 S L5 AND L1
L7 935 S L2 AND L3
L8 15916 S L6 AND 7
L9 497 S L6 AND L7
L10 33 S L9 AND (CALIBRAT? (P) CURVE?) AND (CONTROL? OR STANDARD? OR
L11 33 DUP REM L10 (0 DUPLICATES REMOVED)
L12 32 S L11 AND L4
L13 32 DUP REM L12 (0 DUPLICATES REMOVED)
L14 497 S L9 AND (CONTROL? OR STANDARD? OR REFERENCE? OR KNOWN)
L15 33 S L9 AND (CALIBRAT? (P) CURVE?)
L16 492 S L14 AND (HER? OR ESTROGEN? OR PROSTATE? OR PSA OR EGFR OR AKT
L17 492 S L14 (6P) (HER? OR ESTROGEN? OR PROSTATE? OR PSA OR EGFR OR AK
L18 47 S L17 AND ((OPTICAL DENSITY) AND (IMAGE ANALYSIS))
L19 46 DUP REM L18 (1 DUPLICATE REMOVED)
L20 8712 S 11 (6P) L5
L21 3617 S 12 (6P) L3
L22 425 S L21 (6P) L20
L23 375 S L22 (6P) (HER? OR ESTROGEN? OR PROSTATE? OR PSA OR EGFR OR A
L24 14 S L23 AND (CALIBRAT? (P) CURVE?)
L25 14 DUP REM L24 (0 DUPLICATES REMOVED)

L13 ANSWER 16 OF 32 USPATFULL

ACCESSION NUMBER: 2000:9693 USPATFULL

TITLE: Methods and compositions for screening for or
modulating a tumor associated antigen

INVENTOR(S): Kinders, Robert J., Woodinville, WA, United States
Enfield, David L., Bothell, WA, United States
Hass, G. Michael, Issaquah, WA, United States

PATENT ASSIGNEE(S): Bard Diagnostic Sciences, Inc., Redmond, WA, United
States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6017703		20000125
APPLICATION INFO.:	US 1997-824692		19970408 (8)

	NUMBER	DATE
PRIORITY INFORMATION:	US 1996-15083P	19960409 (60)
	US 1997-38614P	19970306 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Scheiner, Toni R.
LEGAL REPRESENTATIVE: Seed and Berry LLP
NUMBER OF CLAIMS: 9
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 20 Drawing Figure(s); 15 Drawing Page(s)
LINE COUNT: 2828

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Methods of screening for or treating cancer are disclosed. The screening methods are based on the detection of an antigen, or a nucleic acid molecule encoding the antigen, found by the present invention to be associated with the presence of cancer. Preferred embodiments of the methods include detection of the antigen based on immunological properties, physical properties, enzymatic properties and combinations thereof, or detection of a nucleic acid molecule encoding the antigen based on nucleic acid amplification.

SUMM The present invention is generally directed toward screening for or modulating a **tumor** associated antigen. The invention is more particularly related to detecting a complement Factor H-related **protein**, or a nucleic acid molecule encoding such a **protein**, associated with the presence of **cancer**, and to modulating the presence or activity of such a **protein**.

SUMM The detection of new **tumors** or the recurrence of **tumors** remains an unfulfilled goal of humankind, despite enormous expenditures of both financial and human resources over the last twenty-five plus years. A number of **cancers** are treatable if detected at an early stage, but unfortunately go undetected in many patients for lack of a reliable screening procedure. For illustrative purposes, background for a particular **cancer**, bladder **cancer**, is described in more detail and is representative of **cancers** in need of new approaches, which the invention disclosed herein provides.

SUMM Bladder **cancer** is the fifth most common **cancer** in the United States. The American **Cancer** Society estimated that a total of 52,000 new cases would be detected in 1994 and that there would be 10,000 deaths resulting from this disease. Bladder **cancer** is more common in men than in women by a ratio of approximately three to one and has been shown. . . Carcinoma of the urinary bladder is the fourth most common malignancy among American men, and the eighth among women. Transitional **cell** carcinoma (TCC) is the most common type of bladder **cancer** representing greater than 90% of all cases. The remaining cases are squamous **cell** carcinomas (7%), adenocarcinomas (2%), and undifferentiated carcinomas

(1%).

SUMM . . . presenting with such symptoms as hematuria or dysuria in the absence of infection undergoes a cystoscopy at which time the **tumor** is visualized. Although this procedure is invasive and unpleasant, it is highly accurate in predicting malignancy and is, thus, considered the gold **standard**. Urine cytology (i.e., the identification of **tumor cells** in voided urine) is also performed, and the combined results of the two methods may lead to an increase in sensitivity over that of cystoscopy alone. This is due to the fact that cytology occasionally allows detection of **tumors** which are not visible during cystoscopy, for example, flat **tumors** of the bladder (TIS) or those in the upper end of the bladder or the upper urinary tract.

SUMM . . . with this procedure removing the apparent lesion as well as providing information as to the grade and stage of the **tumor**. The **tumor** is typically graded from G0 to G4 in decreasing state of differentiation. As with most **cancers**, the less differentiated the **tumor** the more aggressive the disease. With respect to stage or extent of invasion, TCC's of the bladder may be classified as superficial papillary (Ta and T1), muscle invasive (T2 and greater), or the relatively uncommon **tumor** in situ (TIS). The extent of invasion dictates the type of therapeutic approach employed and the follow-up procedures to monitor.

SUMM Approximately 75% of TCC patients are initially diagnosed as having either Ta or T1 disease. In part because bladder **cancer** is multifocal, initial resection and treatment of these patients is curative in less than half of the cases. Although patients presenting with Ta TCC usually recur, their **tumors** tend to be low grade, and only 10-15% of the **tumors** will progress to muscle invasive disease. In contrast, T1 patients will progress 30-50% of the time. Superficial TCC is usually.

SUMM . . . and some invasive TCC by cystoscopy and urine cytology. Recurrence, especially within the first 12 months, is common, even when **tumors** have been diagnosed and treated prior to invasion of the bladder muscle. Therefore, patients with superficial TCC are typically monitored.

SUMM These and other aspects of the present invention will become evident upon **reference** to the following detailed description and attached drawings.

DRWD . . . 700, 500, 400, 300, 200, 100, and 50 base pairs. Lanes 8 and 9 are PAW 109, the kit positive **control**, at the expected size of 311 base pairs.

DETD As noted above, the present invention is directed, in one aspect, toward methods of screening for **cancer**. As disclosed in the present invention, a **protein** antigen has been found to be associated with the presence of **cancer** ("**tumor-associated**") and found to survive in detectable concentrations in samples from warm-blooded animals, such as humans. The present disclosure describes, for example, the purification of a **tumor-associated** antigen from **cancer** patients, the generation of antibodies to the antigen, the characterization of the antigen by physical and biological properties, the development of **immunoassays** and non-**immunoassays** for the detection of the antigen or a nucleic acid molecule encoding the antigen, the evaluation of samples from normal individuals and **cancer** patients, demonstration of the production of the antigen by **cancer cells**, the determination that the antigen corresponds to **protein** products related to human complement Factor H, and the inhibition of biological activity of the antigen.

DETD A wide variety of **cancers** may be screened. Representative examples of such **cancers** include urogenital, renal, head/neck and lung. Urogenital **cancers** include bladder, cervical and prostate. Head/neck **cancers** include **cancers** of the oral cavity, mouth and esophagus. As used herein, the term "screening

for" includes detecting, monitoring or diagnosing. It will be evident to those in the art that if one wishes to screen for a particular type of **cancer**, this choice will guide the selection of a particular source of **cell**, tissue or sample to be tested. A sample in general may be a liquid or solid (e.g., **cellular**) sample taken from a tissue or organ, or after having been in contact with a tissue or organ. For example, . . .

DETD The detection, isolation, characterization and identification of a **protein** antigen present in specimens derived from patients with **cancer**, but absent in specimens from normal individuals, indicates that this antigen is either a product of the **cancer cells** or is for some other reason present in specimens from these patients. If the antigen is expressed by **cancer cells**, it may be present in the supernatants taken from cultured human **cancer cell** lines at levels adequate to be measured by enzyme **immunoassay** specific for the antigen. cDNA derived by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification from mRNA isolated from the same **cancer cells** can be used as well to provide evidence for expression of the gene which encodes for a product which is. . . similar to the identified antigen. As disclosed herein, both types of experimental approaches confirm the expression of the antigen by **cancer cell** lines (e.g., bladder, cervical, renal and prostate **cancer cell** lines).

DETD The **tumor-associated protein** antigen of the present invention has been determined, by sequence comparisons, surprisingly to be human complement Factor H-related. As **cancer cells** may produce more than one form of the **protein**, as used herein the term "human complement Factor H-related" refers to the human complement Factor H **protein** and variants thereof. The variants may be the result of mutations, alternate splicing or recombination events that alter nucleic acid molecules encoding human complement Factor H. In general, the amino acid sequence identity between a human complement Factor H-related **protein** from a **tumor cell** and human complement Factor H will be at least about 50%. More typically, the amino acid sequence identity will be. . . present in both of the two sequences. In addition, a nucleic acid molecule encoding for a human complement Factor H-related **protein** will typically hybridize under moderately stringent conditions to one or the other or both of two primer pairs (42M/1040RT or 2910M/3610RT), as described below. This reflects conservation of certain sequences (disclosed herein) for **tumor-associated** human complement Factor H-related antigen. A **protein** may generally be identified as a **tumor-associated** human complement Factor H-related antigen based on the ability of a nucleic acid molecule encoding the **protein** to hybridize under moderately stringent conditions to one or the other or both of two primer pairs (42M/1040RT or 2910M/3610RT), as described below. Based on the disclosure herein, in combination with the methodologies **known** in the art, it will be evident to those in the art whether a **protein** is a **tumor-associated** human complement Factor H-related antigen, or whether a nucleic acid molecule encodes such a **protein**.

DETD The antigen may be isolated in substantially pure form. Briefly, for example, urine samples of bladder **cancer** patients are clarified (e.g., by centrifugation) and concentrated (e.g., by hollow fiber concentrator). The concentrated sample is chromatographed on heparin. . . buffered NaCl gradient. Pooled fractions are concentrated. Purity can be assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis ("SDS-PAGE") with appropriate **protein** stains. Alternatively, the antigen may be purified using an antibody against the antigen, as described for example below.

DETD . . . dodecyl sulfate). Approximate molecular weights of polypeptides are assigned by comparison of their mobility to the mobility of polypeptides of **known** molecular weights on SDS-PAGE. Isolated

antigen yields from certain **cancers**, for example, a band with an apparent molecular weight of approximately 151,000 on SDS-PAGE under reducing conditions (i.e., in the. . .

DETD . . . the lymph nodes and/or spleens of an animal injected with antigen in pure or impure form are fused with myeloma **cells** to form hybrid **cell** lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin specific for the antigen and, like the myeloma **cells**, has the potential for indefinite **cell** division.

DETD . . . are harvested from the immunized animals. At this time, the lymph nodes may also be harvested and included in the **cell** preparation. The harvested organs are minced using techniques which disrupt the structure of the organ, but which are not detrimental. . . preparation for lymphocytes. The minced and strained tissue is harvested by centrifugation, then mixed with growth medium to form a **cell** suspension. The red blood **cells** may be lysed by adding a hypotonic or hypertonic solution to the **cell** suspension. A preferred method for **cell** lysis is to add distilled water to the suspensions and quickly return the suspensions to an isotonic state with a. . .

DETD The harvested **cell** suspension is then mixed with a myeloma **cell** line, preferably one which is syngeneic with the immunized animal. Myeloma **cell** lines from various species are widely available through, for example, American Type Culture Collection (ATCC), Rockville, Md. Myeloma **cell** lines commonly used include P3X63Ag8 (ATCC TIB 9), SP2/0-Ag14 (ATCC CRL 1581), FO (ATCC CRL 1646) and 210-RCY-Ag1 (Galfre et. . .

DETD The myeloma cells are cultured in an appropriate mammalian cell growth medium, a variety of which are generally **known** in the art and available from commercial sources. Mammalian cell lines are routinely grown between 36.degree. C. and 40.degree. C.. . . 6.0 and 8.0, preferably about pH 7.2. pH may be maintained through the use of a variety of buffer systems **known** in the art. A preferred buffer system involves growing the cells in a bicarbonate buffer in a humidified incubator containing. . .

DETD . . . At various times following the culturing of the lymphocytes in vitro, the lymphocytes are harvested and fused with a myeloma **cell** line as described above.

DETD Other techniques for producing and maintaining antibody secreting lymphocyte **cell** lines in culture include viral transfection of the lymphocyte to produce a transformed **cell** line which will continue to grow in culture. Epstein-Barr virus (EBV) has been used for this technique. EBV transformed **cells** do not require fusion with a myeloma **cell** to allow continued growth in culture.

DETD Thymocytes may be used as a feeder layer to condition the medium for the fused **cells**. Alternatively, peritoneal macrophages or non-immune spleen **cells** may be used as a feeder layer. Another alternative is to use conditioned medium from thymocytes or macrophages. Thymocytes may. . . using scissors to mince the tissue, followed by passage of the tissue through a mesh screen. The minced and strained **cell** material is then harvested by centrifugation. **Cell** suspensions are made using growth medium. Any remaining connective tissue may be removed by filtration through gauze.

DETD At an appropriate time following the day the **cells** are fused, the fused **cells** (hybridomas) are then analyzed for the production of antibody against the antigen. This "screening" can be done by a wide variety of techniques, including Western blot, **ELISA**, immunoprecipitation, effect on biological activity **assays** and immunocytochemical staining. These techniques and others are well described in the literature. (See, for example, J. G. R. Hurrell. . . definition of antibodies of useful reactivity. For example, antigen purified from a biological sample of a patient with a bladder **cancer** may be used in any of the above-named techniques to define antibodies which react, for example, to determinants which are.

DETD Hybridomas which secrete antibodies of interest are maintained in culture. The **cells** are expanded in culture and at the same time may be cloned in such a manner as to obtain colonies originating from single **cells**. This provides for the monoclonal nature of the antibodies obtained from the hybridomas. A wide variety of techniques exist for cloning **cells**, including limiting dilution, soft agar cloning and fluorescence-activated **cell** sorting.

DETD Once clones of **cells** are obtained, they are re-assayed for the production of the antibody of interest. These **cells** are then expanded in culture to allow for the production of larger amounts of the antibody. Methods for expansion of the **cells** include maintaining the **cells** in culture, placement of the **cells** in a bioreactor or other type of large-scale **cell** culture environment, or culturing the **cells** using various agar or gelatin carrier matrices. Antibodies are then isolated from the **cell** culture media.

DETD Antibodies may be purified from conditioned media or ascites fluid by a variety of methods **known** in the art. These methods include ammonium sulfate precipitation, ion exchange chromatography (see Hurrell, *ibid.*) and high pressure liquid chromatography. . . Immunol. Methods 76:157, 1985). A preferred method for purifying antibodies from conditioned media or ascites fluid utilizes a commercially available **Protein A-Sepharose.RTM.** CL-4B column or **Protein G Sepharose.RTM.** (Pharmacia, Piscataway, N.J.; Sigma, St. Louis, Mo.) or ABX mixed ion exchange resin (J T Baker, Phillipsburg, N.J.).. . .

DETD As disclosed herein, the antigen which is found to be associated with the presence of **cancer** may be detected in a wide variety of ways, including by detecting the antigen itself or a nucleic acid molecule. . . .

DETD . . . measured is inversely proportional to (i.e., indirectly reflective of) antigen present in a sample. Indirect formats include competitive and inhibition **assay** formats. As used herein, the term "antibody" includes both polyclonal and monoclonal antibodies; and may be an intact molecule, a . . . nitrocellulose or Immobilon or similar matrix, in conjunction with specific antibodies to the antigen. Detection can also be achieved by **immunoassay**. In one embodiment, antigen is isolated from a sample and contacted with an appropriate detection antibody. Antigen may be isolated. . . .

DETD . . . or more immunocomplexes formed between antigen and an antibody specific for the antigen may be accomplished by a variety of **known** techniques, including **radioimmunoassays** (RIA) and enzyme linked **immunosorbent assays** (**ELISA**).

DETD The **immunoassays** **known** in the art include the double monoclonal antibody sandwich **immunoassay** technique of David et al. (U.S. Pat. No. 4,376,110); monoclonal-polyclonal antibody sandwich **assays** (Wide et al., in Kirkham and Hunter (eds.), **Radioimmunoassay Methods**, E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Pat. No. 4,452,901); immunoprecipitation of labeled ligand (Brown et al. , J. Biol. Chem. 255:4980-4983, 1980); enzyme-linked immunosorbant **assays** as described by, for example, Raines and Ross (J. Biol. Chem. 257:5154-5160, 1982); immunocytochemical techniques, including the use of fluorochromes. . . . 1980); and neutralization of activity (Bowen-Pope et al. , Proc. Natl. Acad. Sci. USA 81:2396-2400, 1984). In addition to the **immunoassays** described above, a number of other **immunoassays** are available, including those described in U.S. Pat. Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

DETD For detection purposes, the antibodies may either be labeled or unlabeled. When unlabeled, the antibodies find use in agglutination **assays**. In addition, unlabeled antibodies can be used in

combination with other labeled antibodies (second antibodies) that are reactive with the. . . fluorophores, enzymes, luminescers, or visible particles (e. g. , colloidal gold and dye particles). These and other labels are well **known** in the art and are described, for example, in the following U.S. Pat. Nos. 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

DETD Typically in an **ELISA assay** the target antigen (for a competitive or inhibition **assay** format) or immobilized capture antibody is adsorbed to the surface of a microtiter well. Residual protein-binding sites on the surface. . . washed to remove unbound conjugate, and the substrate for the enzyme is added. Color is allowed to develop and the **optical density** of the contents of the well is determined visually or instrumentally.

DETD . . . the presence or absence of the reporter group. An example of a molecule capable of binding to the immunocomplex is **protein A**.

DETD As disclosed herein, this antigen, which is associated with the presence of **cancer**, is bound by complement factor fragment C3b. Therefore, C3b may be used in **assays** (such as those described above) that utilize a capture molecule and a detection molecule for detecting antigen. For example, C3b. . . presence of antigen may be determined by contacting a sample (suspected of containing antigen) with C3b and Factor I, and **assaying** for the digestion of C3b. In the presence of antigen and Factor I, the C3b .alpha.' fragment at a molecular. . .

DETD . . . the level of ordinary skill in the art. With respect to PCR, for example, the method may be modified as **known** in the art. Transcriptional enhancement of PCR may be accomplished by incorporation of bacteriophage T7 RNA polymerase promoter sequences in. . .

DETD . . . in greater detail below) of about 18 to 30 nucleotides are preferred, and can be easily synthesized using techniques well **known** in the art. PCR products, and other nucleic acid amplification products, may be quantitated using techniques **known** in the art (Duplaa et al., Anal. Biochem. 212:229-236, 1993; Higuchi et al., Bio/Technology 11:1026-1030).

DETD A preferred embodiment involves **assaying** for the presence of specific messenger RNA (mRNA) encoding the antigen. More specifically, for example, as described herein, a **cell** sample may be lysed and the mRNA isolated, amplified and examined for the presence of mRNA specific for the antigen.. . .

DETD Detecting the presence of antigen in a **cell**, tissue or sample has a variety of uses. For example, the present invention may be used for diagnostic purposes to screen warm-blooded animals, such as humans, for **cancer** (or a particular **cancer** depending upon the source of the particular **cell**, tissue or sample). Similarly, the present invention may be used to monitor warm-blooded animals. In particular, a preferred use is to follow patients who have been previously diagnosed and treated for **cancer**. Patients who are in remission (or may in fact be cured) can be monitored for the reappearance of **cancer**. It will be evident to those in the art that it may be desirable to use the present invention in conjunction with one or more other tests for **cancer** (or a particular **cancer**) to confirm positive or negative results obtained from use of the present invention.

DETD The unexpected presence of a complement Factor H-related **protein** in **cell** culture supernatants from epithelial **cancer** **cells** (Example VI, Table 7), and the demonstration that its mRNA is produced by **cancer** **cells** (Example VI, Table 7), suggest that it plays a significant role in **cancer** biology. Data presented below (Example III.F) demonstrate that a biological activity of the antigen is to accelerate the complement Factor. . . in vivo is the assembly of the membrane attack complex (MAC) prior to lysis of an appropriate target. Because these **proteins** are members of the Alternative Complement Pathway, **cell** lysis may take place independent of the presence of circulating antibodies to any

of the **cancer cell** antigens. Although not wishing to be bound by theory, in view of the activity of the antigen described herein, its production by **cancer cells** may locally promote the degradation of C3b, thereby inhibiting the formation of the MAC and preventing **tumor cell** lysis by complement. Since the production of the antigen by **tumor cells** may afford a survival advantage, interrupting the production of the antigen or blocking its decay accelerator activity restores susceptibility of the **tumor** to complement-mediated **cell** lysis, thus offering a new approach to **cancer** therapy.

DETD Irrespective of the exact function(s) of the complement Factor H-related **protein** in **tumor** biology, the present invention provides for the modulation of the antigen as a means for treating **cancers**. It will be evident to those of ordinary skill in the art that the antigen may be modulated in a . . . technology. Typically, the complement Factor H antisense DNA is inserted into an appropriate vector (virus) which delivers it to the **tumor cells**. Once inside the target **cells**, the antisense construct would specifically bind to mRNA coding for the complement Factor H-related **protein**, thereby preventing its translation. Primary among the other methods which could be used to interrupt production of the antigen would. . . use of specific molecules which block the transcription of the specific gene or genes coding for the complement Factor H-related **protein**. Chemicals designed to block the ability of the **tumor cell** to produce antigen would preferably be delivered in the vicinity of the **tumor**, rather than systemically, since systemic introduction of such materials could decrease the normal production of complement Factor H by the. . . regulate complement activity. In modulation of antigen production, it is desired to eliminate the production of all complement Factor H-related **protein** by **tumor cells**.

DETD . . . blocks its ability to degrade C3b--is presented as a representative example of modulation of antigen activity as an approach to **cancer** therapy (Example VII). In this example, certain antibodies which bind antigen are shown to accelerate the complement-mediated lysis of rabbit red blood **cells** and HL-60, a human **tumor cell** line. With these reagents, as with those described above, delivery should preferably be administered to the **tumor** site, rather than systemically. For the antibodies described above, reagent affinities should be at least about 10.sup.6 liters/mole and doses. . . within the range of about 0.01 .mu.g/kg body weight to 10 mg/kg body weight. In addition, the preferred type of **tumor** to be treated in this manner would be distinctly separate from the circulatory system, since blood itself contains high concentrations. . . may be replaced by, or supplemented with, any peptide or other organic molecule which specifically binds to complement Factor H-related **protein** and blocks its biological activity.

DETD The antigen source for immunization was a pool of Heparin-Agarose fractionated urines from clinically diagnosed bladder **cancer** patients. (The purification method is described in detail in Example III.A.1. below.) Twenty-four hour urine samples were centrifuged in. . . a JA-10 rotor at 6,000 rpm for 20 minutes. The clarified urine sample was then concentrated using an Amicon stirred **cell**, 76 mm, (cat# 5124) fitted with a YM30 membrane MWCO 30,000 dalton (Amicon, cat# 13742) or a Microgon hollow fiber. . . and fractions from the trailing half of the elution peak were pooled. Pooled fractions were concentrated with an Amicon stirred **cell**, 43 mm (cat# 5122), fitted with a YM30 membrane, MWCO 30,000 dalton (cat# 13722). Fractions comprising the pooled antigen are. . .

DETD . . . II in Freund's Complete Adjuvant (Difco, Detroit, Mich.). Three weeks later, booster immunizations of 0.1 mL containing 10 .mu.g of **protein** of an emulsion in incomplete Freund's Adjuvant was administered to the rear footpads and peritoneum. Ten days later each

mouse was sampled for antibody response via retro-orbital bleeds and the sera were tested via an **ELISA** described below for titers. Mouse number 340 showed the highest titer and was chosen for fusion four days after boosting. . . .

DETD . . . for fusion, at a ratio of one to five lymphocytes. PEG-DMSO (Sigma, St. Louis, Mo.) fusogen, was used, and the **cells** plated out in Iscove's Modified Dulbecco's Medium (IMDM) with penicillin-streptomycin and hypoxanthine/thymidine (HT) supplement at a density of 2.times.10.sup.4 **cells**/well with 2.58.times.10.sup.3 peritoneal macrophages from unimmunized BALB/C mice added as feeders. The fusion was divided into two parts, in the. . . .

DETD D. Post-Fusion Cell Culture

DETD Wells selected via the screening **assays** were immediately transferred to 24 well plates containing 1 mL of complete IMDM containing 10% FBS. A sample of **cells** was also used to immediately re-clone the hybridomas by a serial limiting dilution procedure. This consisted of transferring a 10 .mu.l sample of **cells** from the chosen well of the 96 well plate to the first well of a fresh 96 well plate previously. . . . of complete IMDM with 10% of a cloning supplement prepared from murine macrophages and thymocytes (Condimed, Boehringer-Mannheim Corp., Indianapolis, Ind.). **Cells** from the first well were serially diluted in the first column of wells by transferring 100 .mu.l from the first. . . . well. The wells of the first column were then serially diluted across the plate by transfer of 50 .mu.l of **cell** suspension using an 8 place pipette. Finally, 100 .mu.l of cloning media was added to each well, and the plates. . . . with 5-6 mL of culture media, the plates were incubated until near confluent growth was observed. A sample of the **cells** were removed for storage in a cryogenic freezer in 5% DMSO in FBS, and the remaining **cells** were transferred to a T-75 flask with 10 mL media for producing spent media for further testing.

DETD Subclones were again subjected to testing via **ELISA** (described below) incorporating an additional urine from a patient diagnosed as TCC+. Typically all subclones of a given original-evaluated well. . . .

DETD F. Assays

DETD The titer **assay** was carried out by coating Pool II (Example I.A., above) antigen adjusted to 4 .mu.g/mL in 0.1 M carbonate buffer,. . . . for 1 hour at 37.degree. C. The plate was washed with PBS 4 times, and 50 .mu.l of substrate (K-Blue, **ELISA** Technologies, Lexington, Ky.) was added and the plate allowed to develop for 10 minutes at room temperature before stopping the reaction via the addition of 100 .mu.l of 2M phosphoric acid solution in water (Sigma). The **optical density** of the wells were read at 450 and at 410 nm in a BioTek EL311 plate reader. Readings which were. . . .

DETDmu.l of diluent was added, and 50 .mu.l was distributed to the test plate wells. The remaining steps of the **assay** were as for the titer **assay**, with the exception that the conjugate used was human serum adsorbed goat anti-mouse IgG-HRP conjugate (Kirkegaard and Perry Labs (KPL),. . . . alkaline phosphatase conjugate of a similar antibody was used (KPL, Gaithersburg, Md.) followed by use of PNPP (p-nitrophenyl phosphate) substrate. **Controls** were used for each **assay**, negative **control** was fresh IMDM with 10% FBS, positive **controls** were monoclonal anti-human collagen (Sigma C1926), and monoclonal anti-hIgA (A1.1.2.4, Bard Diagnostic Sciences, Inc., Redmond, Wash.), both of which showed high binding to all test antigens except the red blood cells. Criteria for selection were high binding to **cancer** urine plates (OD>1), low binding to normal urines and other test antigens (OD<0.5). Others which showed high antibody levels in different patterns with respect to the test antigens were also selected for potential. . . .

DETD Subclones were screened by several **assays**. First, the fusion **assay** was again used then, following expansion in culture of selected subclones, an abbreviated **ELISA** was employed using normal urine pool I and the two advanced stage urines used in the fusion

assay. The testing was carried out at dilutions of 1:10 and 1:100 for the early subclones, and an additional dilution of 1:1000 for the later subclones. In several of the subclone **assays** the addition of urine from a patient with a lower grade **cancer** was included.

DETD . . . the 118 clones selected, 37-X and 8-I series were eventually lost due to instability or lack of growth without feeder **cells**

DETD A total of 32 subclones were selected based on selectivity of antibody binding to **cancer** positive urines versus the normal urines and on retention of **assay** OD with dilution of culture supernatant to select for high affinity and good production level. Samples of spent culture media from the following clones were evaluated for their potential utility in a clinical **assay** for the antigen described in Example III: I-7.3, I-8.2, I-10.2, I-11.1, I-12.2, I-17.3, X-4.1, X-13.1, X-13.2, X-22.2, X-28.1, X-44.1, X-49.1, . . . X-55.1, X-56.3, X-59.1, X-60.2, X-61.2, X-62.1, X-63.2, X-64.3, X-67.2, X-69.1, X-70.2, X-84.2, and X-87.2. A preferred monoclonal antibody pair for **assays** is X-13.2 (conjugate MAb) and X-52.1 (capture MAb).

DETD Heparin-Agarose chromatography (Example I.A., above) fractions from three TCC-positive patients were pooled and dialysed against phosphate buffered saline (PBS). **Protein** concentration was determined to be 2 mg/mL. Thimerosal was added to a final concentration of 0.02%, and 0.25 mL aliquots were frozen until use. Table 1 is a listing of the amounts and **references** of the antigens comprising Pool I.

DETD Serum samples were taken pre-immunization and two weeks after the second and third immunizations and were analyzed via **ELISA** using the antigen coated onto microplates. The **assay** was similar to the **ELISA** used for the mouse serum titer with the exception that antigen Pool I and rabbit anti-goat IgG-HRP were used and. . .

DETD . . . a JA-10 rotor at 6,000 rpm for 20 minutes. The clarified urine sample was then concentrated using an Amicon stirred cell, 76 mm, (cat# 5124) fitted with a YM30 membrane MWCO 30,000 daltons (Amicon, cat# 13742) or a Microgon hollow fiber. . . and fractions from the trailing half of the elution peak were pooled. Pooled fractions were concentrated with an Amicon stirred cell, 43 mm (cat# 5122), fitted with a YM30 membrane, MWCO 30,000 daltons (cat# 13722).

DETD 2. **Protein A** Chromatography of 24 Hour Urine

DETD **Protein A** Chromatography was performed on a 24 hour urine from a TCC+ patient to determine whether this **tumor** antigen could be part of an immune complex. The urine (6 mL) was diluted to 12 mL with the addition. . . 6 mL of 20 mM sodium phosphate, pH 7.4. The diluted urine (7.3 mL) was loaded on a 1.0 mL **Protein A** cartridge (BioRad, Richmond, Calif., cat# 732-0093) equilibrated in 20 mM sodium phosphate, pH 7.4, at 0.5 mL/min. The flow. . . load, flow through, and eluted pool, at dilutions of 1:20 to 1:2560, were tested in the double monoclonal microtiter plate **assay** described in detail below (Example IV.B.). Approximately 97.5% of the activity loaded was contained within the flow through peak. The. . . to incomplete washing. Thus, this antigen is not part of an immune complex involving IgG, and the use of immobilized **Protein A** would not be effective in extracting the antigen from specimens.

DETD . . . MAb affinity columns (BioRad A10 gel) prepared with MAb X-13.2 or MAb X-52.1 (Example I.F.). To serve as a **control** for urine materials binding non-specifically to IgG, an A10 **control** column was prepared using **Protein A**-purified, normal mouse serum. Samples were loaded at 0.5 mL/min. The sample was eluted with 25 mM Tris-HCl, 250 mM. . . and/or 4-12% polyacrylamide gels under reducing and non reducing conditions, along with Novex (San Diego, Calif.) Mark XII molecular weight **standards** (6 to 200 kD). The gels were stained with Coomassie Blue R250 followed by silver staining and scanned using a BioRad GS7000 densitometer. Molecular weights of individual bands are estimated based on the Rf values of the molecular weight **standards** (Example III.C.).

DETD A set of gel filtration **standards** (BioRad cat# 151-1901), with a range of 1.3 to 670 kD was dissolved in column equilibration buffer (PBS), filtered through. . . nm wavelength was recorded at 2.0 cm/hr. A high molecular weight aggregate eluted at the column void volume (Vo). Each **protein** peak had its elution volume (Ve) determined by multiplying the time of elution of the maximum absorbance by the flow rate. A linear **calibration curve** was generated by graphing the Ve/Vo of the **standard proteins** vs. the logs of their molecular weights. Molecular weight estimates of the samples' peaks were made using the linear equation generated by the **calibration curve**.

DETD A twenty-four hour urine from a TCC+ patient was concentrated using an Amicon stirred **cell**, fitted with a 43 mm, YM30 membrane (MWCO 30,000 dalton) (cat# 5122). The urine was concentrated 300.times. to .about.0.5 mL. . . S300 column at 0.7 mL/min and 7 minute fractions were collected. The individual fractions were tested in the double monoclonal **assay** (described in Example IV.B.) to detect the presence of the antigen. A range of native molecular weights for the active. . .

DETD . . . the presence or absence of dithiothreitol (DTT; reducing sample buffer), onto a Novex discontinuous, 8% polyacrylamide, two well gel. A **reference** well contained Novex Mark XII SDS-PAGE molecular weight (MW) **standards**. Gels were electrophoresed at 125 V constant until the sample front reached within .about.0.5 cm of the bottom of the gel. The gel's **protein** bands were then transferred to PVDF and stained with Coomassie Blue R250.

DETD Rf values were calculated for the Mark XII individual molecular weight **standards** by dividing the distance the band moved through the resolving gel by the distance of the sample front from the top of the resolving gel. A linear **standard** curve was established by plotting Rf values versus log MW for each MW **standard**. Sample bands' molecular weights were estimated by calculating their Rf values and entering these values (yi) in the **standard** curve equation.

DETD . . . Only bands at 151, 130, and 39 kD appeared to be specific for the MAb X-52.1 in that the other **proteins** also bound to immobilized non-specific mouse IgG. Of these bands, that corresponding to a molecular weight of 151 kD is. . . a large number of intra-chain disulfide bonds in these molecules. This characteristic electrophoretic behavior formed the basis for the antigen **assay** described in Example IV.F.

DETD . . . a Novex electrophoresis chamber (Novex, cat# EI9001) and a BioRad Power Unit 500 V (cat# 165-4710). Novex SeeBlue Molecular Weight **Standards** (cat# LC5625) were loaded into a **reference** well. The SDS-PAGE bands were transferred to PVDF paper (Novex, cat# LC2002) in Novex Transfer buffer (cat# LC 3675) using. . .

DETD . . . a Novex electrophoresis chamber (Novex, cat# EI9001) and a BioRad Power Unit 500 V (cat# 165-4710). BioRad SDS-PAGE Molecular Weight **Standards** (cat# 161-0317) were loaded into a **reference** well.

DETD A **known** quantity of antigen was added to a clean 600 .mu.L Eppendorf tube and PBS added to bring the concentration to. . .

DETD . . . GelDry solution (NOVEX, San Diego, Calif.) for twenty minutes and then dried in between two sheets of DryEase mini precut **cellophane** (NOVEX, San Diego, Calif.) overnight. Seven fragments were observed.

DETD . . . Louis, Mo.) containing 10% methanol (ACS grade reagent), pH 11.0 (blotting buffer) to remove any contaminants from the gels. The **proteins** were transferred onto PVDF membranes (NOVEX, San Diego, Calif.) by electroblotting in blotting buffer at 30 V constant for 1.0.

DETD . . . AA 324 .sup.3 RPYFPVAVGKY

NO: (8)

[RPYFPVAVGKY] (NO:8)

.sup.1 Amino acid residue numbers refer to the mature CFH molecule
 .sup.2 Major **protein** sequence
 .sup.3 Minor **protein** sequence

DETD . . . Factor H (shown in brackets) demonstrates that the antigen detected is a member of a complement Factor H-related family of **proteins** as disclosed herein.

DETD . . . hour, into GelDry solution (NOVEX, San Diego, Calif.) for twenty minutes and dried between two sheets of DryEase mini precut **cellophane** (NOVEX, San Diego, Calif.) overnight.

DETD The dried gel was analyzed with a BioRad Model GS-700 **Imaging** Densitometer equipped with BioRad's Molecular Analyst software and the molecular weights of the digestion fragments were estimated using Mark12 molecular weight markers as **standards**.

DETD . . . This digestion only occurs when mediated by a cofactor molecule, such as Complement Factor H, as was apparent in the **control** runs. For example C3b, when incubated with Factor I alone, does not degrade. Urine affinity purified antigen mediated the digestion. . .

DETD **ASSAYS FOR THE ANTIGEN**

DETD . . . the antigen as described above and given the disclosure herein for generating and selecting antibodies and the development of certain **assays** described herein to detect the antigen, a number of additional **assay** formats beyond those described herein for this antigen may be readily developed by those of ordinary skill in the art. Suitable **assay** formats include competitive formats, sandwich formats (Examples IV.A., IV.B. and IV.C), **assays** based on the biological or chemical properties of the antigen (Example IV.D. and IV.E.), **assays** based on the simultaneous binding of the antigen to a specific macromolecule (e.g., C3b) and to a monoclonal antibody (Example IV.D.), **assays** based on the appearance of a band of appropriate size in partially-purified specimens (Example IV.F.), and RT-PCR (Example IV.G.). A preferred format involves sandwich **immunoassays** and the most preferred employs a monoclonal antibody immobilized on a solid surface and a second monoclonal antibody, which recognizes. . . agent could be an enzyme (Example IV.B.), colloidal gold (Example IV.C.), or any of a number of other such agents **known** to those of ordinary skill in the art. These include fluorescent molecules, radioisotopes, and biotin (which would subsequently bind to. . .

DETD Initial screening of the cell culture supernatants (Example I.F.) was carried out using an **ELISA** in an indirect format. The assay consisted of the following in order: (1) diluted urine samples were adsorbed on a. . .

DETD . . . number of urine samples in this manner, all seven were selected for further study in the sandwich format of the **ELISA**.

DETD . . . polyclonal preparation (Example II) were tested as capture antibodies in combination with the seven alkaline phosphatase conjugates in the sandwich **ELISA** format as follows: (1) individual capture antibodies were adsorbed on microtiter plates; (2) following washing, diluted urine samples were added. . .

DETD B. Sandwich **ELISA**

DETD The sandwich **ELISA**, utilizing the most preferred pair as selected above, was further optimized with respect to the following items: (1) coating level. . . of capture antibody; (2) concentration of conjugate; (3) enzyme-to-antibody ratio in the conjugate; (4) reaction kinetics/incubation times; (4) composition of **assay** and wash buffers and of conjugate and specimen diluents; and (5) formulation of **standards** and **controls**. The **assay** as optimized is performed as follows:

DETD Antibodies were purified by chromatography on immobilized **Protein G** or **Protein A** by **standard** techniques. Although antibody-enzyme conjugates could be prepared using a variety of coupling techniques (for review see Scouten, W. H., Methods. . .

DETD 3. **Assay Format**
DETD A volume of 175 μ l of **assay** buffer was pipetted into each well to be utilized in carrying out the **assay**. The buffer was followed by 25 μ l of samples, **standards**, or **controls**, thus yielding a 1/8 dilution in the well. Incubation of the covered plate was performed at 37.degree. C. for 60. . .

DETD Eighty seven urine samples were **assayed** by the **ELISA** using the format described above. These samples included 23 clinical specimens taken from patients diagnosed as currently having transitional cell carcinoma (TCC) and 64 others. The results are tabulated below in Table 4. Sensitivity is reported as the percentage of specimens from TCC-positive patients that correctly produce a positive result in the **assay**. Specificity is reported as the percentage of urines from individuals without TCC that correctly produce a negative result in the **assay**.

DETD It is clear that the **ELISA** described here for the detection of this **tumor** antigen yields a positive reaction with a significant number of urine specimens taken from patients diagnosed with bladder TCC. Samples. . .

DETD C. Rapid **Assay**
DETD Monoclonal antibodies specific for the antigen (Example I.F.) were utilized in a lateral flow format to produce a qualitative **assay** for bladder **cancer** using urine as the specimen. The lateral flow format consisted of a colloidal gold antibody conjugate and an immobilized capture. . . Material not bound by the capture antibody continued to migrate through the membrane and contact an immobilized goat anti-mouse antibody (**control** zone) which bound the colloidal gold conjugate regardless of the presence of antigen, forming a visually detectable signal in the **control** zone.

DETD An airbrush sprayer was used to immobilize the capture and **control** antibodies on the membrane. Purified monoclonal antibody X-52.1 at 2 mg/ml was sprayed as a line onto a section of. . .

DETD . . . result will show a pink-purple line in the test zone (zone of immobilized X-52.1) and a pink-purple line in the **control** zone (zone of immobilized goat anti-mouse). A negative result will show no line in the test zone and a pink-purple line in the **control** zone. The absence of a line in the **control** zone indicates that the reagents in the test did not function properly and this test is invalid. Twenty three TCC-positive. . .

DETD . . . in 50 mM carbonate buffer, pH 9.6, either overnight at 4.degree. C. or for two hours at 37.degree. C. A **control** plate was coated with 50 μ l per well of 2% BSA in PBS for two hours at 37.degree. C. After. . .

DETD . . . according to manufacturer's protocol. Reduced and non-reduced samples were run on the same gel and were separated by molecular weight **standards** and an empty lane loaded with non-reducing 1.times. sample buffer. The gels were stained with Coomassie Blue R250 0.1% in. . .

DETD . . . amplification of messenger RNA, mRNA), a variety of procedures used to detect the presence of specific RNA can be used. **Controls** were performed using PCR target materials (the PAW109 sequence) provided with commercial PCR kits, and its primers DM152 and DM151. . . cell line X-44.1 or normal human epithelial keratinocytes (Clonetics Corp., San Diego, Calif.) were chosen as the irrelevant target (**Negative controls**).

DETD . . . of the expected size, 341 bp, upon staining with ethidium bromide (FIG. 2, lane 3). Amplification of the kit positive **control** PAW 109 gave the expected 311 base pair product. Re-amplification of this product with the kit DM152 and 151 primers. . .

DETD Results from the study of the cervical specimens with this **assay** are tabulated in Table 6 and presented graphically in FIG. 12.

DETD TABLE 6

Sensitivity (**cancers**) n = 15
73%

Specificity (normals) n = 19 100%

% Adenocarcinoma above cutoff n = 3 33%

% Dysplasia above cutoff n = 19 16%

% Atypicals above cutoff n = 7 0%

Cutoff (Mean OD of Normals + 2SD) 0.201

DETD PRODUCTION OF CFHRP IN **CANCER**

DETD 5 A. Production of CFHrp in **Cancer Cell Lines**

DETD 1. Detection of Antigen in **Cell Culture Media** by Immunossay

DETD **Cell culture media** were tested for the presence of antigen (complement Factor H-related **protein**, CFHrp) using the sandwich enzyme **immunoassay** as described in Example IV.B. The media tested were those taken from **cell cultures** used for the preparation of total **cellular RNA**. After removal of the cultured **cells**, the remaining media free of **cells** were then diluted, as necessary, and tested in the EIA, as described. **Control** experiments involved the testing of fresh media, in particular those specified by ATCC or Clonetics Corporation (San Diego, Calif.) for the **cell lines** or primary cultures of interest. These were typically Modified Eagle's Media containing 10% fetal bovine serum (Sigma Chemical).

DETD 2. Detection of Message for Antigen in **Cancer Cells** by RT-PCR

DETD cDNA was synthesized from mRNA present in preparations of total **cellular RNA** from **cancer cell lines**, using Reverse Transcriptase plus Random Hexamer primers. The concentrations of components within the reaction mixture were as follows:

DETD . . . melting temperature (Tm) of the probe/primer with the target. The recommended formula for calculating Tm, and its limitations, are well **known** in the art (i.e., are found in Sambrook, J., Fritsch, E. F. and T. Maniatis, Molecular Cloning, 2d Edition, Cold. .

DETDmu.L of the purified material on a 2% agarose gel (Sigma) and comparing its intensity to the intensity of a **standard** (Sigma) of **known** concentration.

DETD f. A specificity **control** was performed by application of riboprobe stock containing a 100-fold excess of riboprobe that had not been labeled with digoxigenin.

DETD The target tissues subjected to staining with the riboprobe were serial sections from normal and **cancerous** human bladder (transitional **cells**) and from normal and **cancerous** human prostate. All tissue sections, both normal and **cancerous**, were from a single bladder or a single prostate.

DETD . . . was established by competition of digoxigenin labelled probe binding with a 100-fold excess of unlabelled probe. Only sections from TCC+bladder **cancer** stained with the HeLaS3 generated probe sequence.

DETD A. In Vitro Protection of C3b by Anti-CFH Related **Protein MABs**

DETD . . . clarity, therefore, Factor H and Factor I were used to degrade C3b and illustrate the protective actions of anti-Factor H-related **protein MABs**.

DETD . . . (BioRad, Hercules, Calif.). The intensities of the bands measured in this way were converted to percentage of C3b remaining. The **control** lane containing the reaction mixture in the absence of MAB was used to represent 100 percent degradation, while the lane. . .

DETD B. Activation of **Cell Lysis** by the Alternate Complement Pathway by MABs Specific for the Antigen

DETD **Standard** guinea pig complement was treated with 5 mM EGTA to chelate calcium. Then 5 mM MgCl.sub.2, which is required for. . .

(ACP), was added. The mixture was incubated for 20 minutes at 37.degree. C., then added to 7.times.10.sup.9 rabbit red blood **cells** (RBC) and further incubated at 37.degree. C. After 45 minutes and again after 117 minutes, hemolysis was determined by measuring the A.sub.450 and comparing the values to those determined for **control** reactions, which had received either no complement or no MAb. Measurements were performed on a Dynatech (Chantilly, Va.) MR5000 96-well. . .

DETD A second experiment was performed under the same conditions as the RBC lysis, but the target **cells** were HL-60 (1.times.10.sup.8 **cells**), a human myeloid **cell** line. MAb concentration was set to 10 nM, and lysis was read after 120 minutes as described above (FIG. 4).

DETD TABLE 9

Inhibition of C3b Degradation in the Presence of Anti-Antigen MABs		
Quantity of		C3b Remaining
Sample	Sample (Percent)	
<hr/>		
Control, No MAb	Standard	0
X52.1	15 .mu.g	0
X52.1	30 .mu.g	0
X87.2	15 .mu.g	24.7
X87.2	30 .mu.g	54.9
X13.2	15 .mu.g	62.0
X13.2	30 .mu.g	54.0
Control, No Factor H		Standard 100

DETD All publications and patent applications mentioned in this specification are herein incorporated by **reference** to the same extent as if each individual publication or patent application was specifically and individually incorporated by **reference**.

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**** See image for Certificate of Correction ****TITLE: Cell analysis method using quantitative fluorescence image analysisAbstract Text (1):

A system for evaluating one or more biochemical markers for evaluating individual cancer risk, cancer diagnosis and for monitoring therapeutic effectiveness and cancer recurrence, particularly of bladder cancer. The system uses automated quantitative fluorescence image analysis of a cell sample collected from a body organ. Cells are treated with a fixative solution which inhibits crystal formation. Cell images are selected and stored as grey level images for further analysis. Cell images may be corrected for autofluorescence using a novel autofluorescence correction method. A neural net computer may be used to distinguish true-positive images from false-positive images to improve accuracy of cancer risk assessment. Cells having images positive for a marker may be compared to threshold quantities related to predetermined cancer risk.

Brief Summary Text (2):

This invention relates to methods for screening cell samples for cytological factors using quantitative fluorescence image analysis, and more particularly, but not by way of limitation, to a method for screening cell samples for cytological factors indicative of cancer or for an increased risk for cancer using quantitative fluorescence image analysis.

Brief Summary Text (6):

Research has shown that an image analysis system can screen urine samples having cells labeled with DNA-binding fluorescent dyes to identify "alarms," which are potentially abnormal objects that exceed certain size-brightness thresholds. When this is coupled with a trained human observer to eliminate artifacts and visually classify cells and with DNA measurements to detect cells that exceed the limit of 5C DNA, an effective cancer detection system results. Because the normal diploid amount of DNA (2C) can be doubled in dividing cells, it is not possible to determine from ploidy alone whether a cell in the 2C-4C region is a normal cell in the process of division or an abnormal cell, additional parameters are needed. Also, morphology alone is insufficient, since many low-grade tumors produce "atypical" cells which have minimally altered morphologies and are also produced by noncancer processes.

Brief Summary Text (8):

The mainstay of cancer diagnosis has been the recognition of cancer cells by a human expert. Humans can learn to recognize such cells visually, but the process of screening samples generally requires a high level of skill and knowledge. The work is generally fatiguing and boring due to its repetitive nature. Cytology is therefore an excellent candidate for automation, and it has been a desired goal to combine quantitative measurements of cell features to identify cancer cells in cell specimens. Although a number of different approaches have been tried, the image analysis approach of attaching a television camera to the microscope, and extracting "features" from the image, and then using those quantitative feature measurements as diagnostic parameters has been used almost exclusively. The term "features" encompasses a wide variety of parameters, including dimensional and ratio parameters. Dimensional parameters include, but are not limited to, density (brightness or darkness), area and length measurements, and dispersion measurements (e.g., standard deviation) of features. Ratio parameters include nuclear/cytoplasmic

area and other similar derived parameters. Image analysis is by and large an algorithmic approach, and ultimately bogs down in the long computational times required to process images and features with discriminant analysis or other statistical approaches.

Brief Summary Text (9):

Quantitative Fluorescence Image Analysis

Brief Summary Text (10):

Quantitative fluorescence image analysis (QFIA) is an instrumentation technology that can be used to quantitate molecular changes at the cellular level. The technology relies on a computerized microscope programmed and standardized to automatically make biochemical and immunochemical measurements at the molecular level in single cells using fluorescent probes. The particular advantage of image analysis is that quantitative molecular determinations can be directly correlated with the wealth of information inherent in visual morphology. Proper standardization, and attention to the fluorescent and stoichiometric properties of dyes are the key to using fluorescence as a quantitative methodology.

Brief Summary Text (18):

Absorption consists of a darker signal imposed upon a bright background. Operationally, $I_{\text{sub.o}}$ is measured by measuring the $##EQU1##$ intensity of light transmitted through the slide in a region where there is no absorbing sample while I is measured after passing through the sample. With an image analysis system, an image of a field is captured, and those areas in the background where nothing is absorbing give a measurement of $I_{\text{sub.o}}$. While absorbance and concentration are linearly related, concentration and the intensity of transmitted light, I , are not. Thus, a logarithmic transformation of data is required in order for results to be accurate.

Brief Summary Text (19):

The amount of substance present is calculated by multiplying the concentration by the volume, V , in which the absorbing material is confined. In theory, the amount of DNA could be calculated from Eq. 2 if the molecular constant a were known and if there is a direct proportionality between the amount of probe bound and the amount of DNA that binds it. The actual image consists of a continuous range of different intensities because DNA is not evenly distributed within the nucleus. Note that the volume is the product of the cross sectional area, A , and the thickness, which is the same as L . Thus, the pathlength, L , disappears from the equations. $##EQU2##$

Brief Summary Text (20):

A digitized image actually consists of discrete "pixels" or picture elements. An example is the discrete dots that comprise an ordinary television image. In digitization, each pixel, which actually represents an average over some small area, is assigned a discrete value, usually between 0 and 256. White would be 0 while completely black would be 256. This value is referred to as the "grey level" and is denoted by the symbol G . The net result is that the continuous variable I is replaced with the discrete variable G . This operation lumps values that are very close to each other together in the same "box" or grey level value, but the human eye is not able to distinguish the digitized signal from the continuous natural one. If the pixel area is $S_{\text{sub.p}}$, then the total DNA content of a cell nucleus is calculated by summing the DNA contained in each volume corresponding to a pixel over all N pixels that comprise the image. This volume is $S_{\text{sub.p}} L_{\text{sub.i}}$, where $L_{\text{sub.i}}$ is the pathlength at the i th pixel. An equation equivalent to Eq. 2 can be derived and is shown as Eq. 3. $##EQU3##$

Brief Summary Text (21):

The summation term is the integrated optical density, or IOD. IOD is time consuming to measure because the logarithmic transformation must be performed on each and every data point. Many systems abbreviate the calculation and do not perform the logarithmic calculation on each pixel element. Instead, they calculate an integrated grey level, IGL, which is the average grey level of the image. $##EQU4##$ With absorbance, an error factor is created when the image is not of uniform density, as is the case of images of cells. The error occurs because intensity of transmitted light and concentration are logarithmically, not linearly, related. For an image

analysis system, the more exact relation shown in Eq. 3 is approximated as described in Eq. 5. The accuracy of the approximation is dependent upon the range of variation in intensities. ##EQU5##

Brief Summary Text (22):

In practice, α is not known and, indeed, varies from assay to assay and batch to batch of samples because of the problem that the chemical methodology is not particularly reproducible. This problem occurs whether a chemical reaction such as the Feulgen procedure is used or stains such as the Papanicolaou stain are used. This problem is partially overcome by using a standard material, for example, a cell type that has a DNA content that is assumed from other measurements. In the normal, resting cell, the amount of DNA is an exact, fixed amount that is arbitrarily assigned a value of 2.0C. In practice, because, (1) some cells may be dividing (and have more DNA), (2) there are certain errors inherent in measuring the OD of an image, and (3) there may be some cell-to-cell variation in staining or labeling, the DNA content of a number of normal cells would be determined and the mode used to set the point 2.0C. The mode is selected because it is relatively insensitive to individual variations in the cells being measured. If one further assumes that the molecular constant for the two cell types will be identical (which is not always true), then the DNA content of an unknown cell type, DNA.sub.u, is related to the DNA content of the standard cell, DNA.sub.s, by Eq. 6, where the term $M(-\log IGL.sub.s / G.sub.0,s)$ refers to the mode of the histogram of OD for the normal cells. ##EQU6##

Quantification by Fluorescence

Brief Summary Text (26):

In the present invention, the background of a fluorescent signal, which is essentially black, is assigned a grey level of zero. The gain of the camera is adjusted such that the usual cell images fall within some range that allows for very bright signals. This arbitrary point is assigned to grey level 255. Thus, any signal brighter than this will be truncated at $G=255$. In the present invention, these truncation occurrences are reported to prevent errors. The real advantage of fluorescence is the linear relationship between fluorescence and concentration using IGL leading to an essentially error-free measurement of amounts of molecules.

Drawing Description Text (2):

FIG. 1 is a schematic of the overall process of cell analysis using quantitative fluorescence image analysis.

Drawing Description Text (3):

FIG. 2A is a grey level image of an abnormal cell labeled with M344.

Drawing Description Text (4):

FIG. 2B is a grey level image of a normal cell labeled with M344.

Drawing Description Text (18):

FIG. 12A is a partial schematic of an Image Analysis System showing main components.

Drawing Description Text (19):

FIG. 12B is a partial schematic of Image Analysis System showing interface subunits.

Drawing Description Text (20):

FIG. 12C is a partial schematic of Image Analysis System showing output, image processor and image memory subunits.

Drawing Description Text (21):

FIG. 13 is a schematic of software-controlled quantitative fluorescence image analysis with G-actin and DNA as markers.

Drawing Description Text (22):

FIG. 14A is a schematic of the first stage of automated scan of slide with image clipping using G-actin and DNA.

Drawing Description Text (23):

FIG. 14B is a schematic of the second stage of automated scan of slide with image clipping using G-actin and DNA.

Drawing Description Text (24):

FIG. 15 is a schematic of software-controlled "rare-event" quantitative fluorescence image analysis using M344 and DNA.

Drawing Description Text (28):

FIG. 18 is a schematic of software-controlled quantitative fluorescence image analysis with DNA and two other markers.

Detailed Description Text (2):

The present invention provides a system for evaluating one or more cytological markers for cell analysis, particularly for individual cancer risk assessment, cancer diagnosis, and for monitoring therapeutic effectiveness and cancer recurrence. Quantitative measurements of phenotypic marker profiles can be used to document the risk of malignancy faced by an individual. While many genetic changes may lead to the malignant phenotype, a much smaller number of phenotypic markers may be used to chart the progress towards malignancy. The current invention represents in one version the first successful application of the neural network approach where the input is a gray level image derived from cells labeled for specific molecules using fluorescent probes.

Detailed Description Text (5):

The term affinity probe as used herein is defined to include a material having a specific affinity for a particular type of cytological marker and may include, but is not limited to antibodies, peptides or polypeptides, nucleotides or polynucleotides, dyes, carbohydrates, lectins, and other ligands, and combinations thereof. Several examples of affinity probes are the M344 antibody, anti-EGFR probes such as AB-1, anti-HER-2/neu protein probes such as TA1, and DNase I.

Detailed Description Text (6):

The slide is analyzed using a quantitative fluorescence image analysis system using a system including a microscope means which automatically selects and stores the grey level images of from about 24 to 115 cells per slide. The term microscope means, as used herein, refers to any means by which cells may be magnified to be viewed at a microscopic level, and may include any viewing means which allows the quantitative measurement of cellular markers within a cell. For example, from a slide double-labeled for actin and DNA, 64 images may be stored for DNA evaluation and 48 images may be stored for actin evaluation. The term actin, as used herein, means any cellular actin-type molecule such as F-actin, G-actin, and any other cytological or nuclear actins. Each cell image may be corrected for extraneous fluorescence including background fluorescence (from the sample medium) and autofluorescence. Cells are then quantitatively analyzed for fluorescence of the specific cytological markers. The term cytological marker is meant herein as any cytological feature which may serve to "mark" a particular type of cell or other component of the cell sample and may include, but is not limited to, tumor associated antigens, receptors, cytoskeletal proteins, oncogene proteins, DNA (including genes and chromosomes) and RNA and which may be labeled by a fluorescent label.

Detailed Description Text (18):

For example, presence of the p300 protein produced by low-grade tumor cells and some dysplastic cells (and detected by the M344 antibody), is a further indicator of risk for cancer. In a study of over 600 urine samples from workers exposed to chemicals that cause bladder cancer, some of whom also smoked, some 15% showed abnormal actin, some 8% showed abnormal DNA and some 2% showed abnormal p300. However, only 5 showed all 3 markers abnormal, and 3 of these were found to have bladder cancer. Of these, 2 had been positive a year earlier, but had not had a detectable cancer at that time. Thus, having all 3 markers positive appears to occur late in the tumorigenesis, near the time when a tumor can be detected, and is a very strong indicator of risk.

Detailed Description Text (24):

FIG. 3 indicates that finding two cells per 10,000 cells examined in a voided urine

is a strong, positive marker for cancer risk. The p300 protein is also expressed by at least some premalignant lesions, as shown by the finding of positive cells in some of the controls. Positive cells were also found in patients with benign prostatic hyperplasia and bladder outlet obstruction, suggesting the protein is a marker for altered differentiation that can be produced either by carcinogenesis or by the promoting effects of urinary stasis. Completely asymptomatic normals express this protein very rarely, and the specificity is in excess of 98% with such individuals. Overall, with the inclusion of symptomatic individuals, the sensitivity with voided urines was 90% with 90% specificity. Preliminary analysis of the data using the stratified risk approach and the field disease studies suggest that p300 appears earlier than abnormal DNA ploidy but after abnormal G-actin.

Detailed Description Text (27):

G-actin has also been investigated as a marker in cancer patients. Globular actin or G-actin, as the monomeric precursor for F-actin, bears a reciprocal relationship to F-actin. Decreased F-actin (a cytoskeletal protein) levels in urinary tract cells is a marker for decreased differentiation. In 9 patients with biopsy-proven disease the mean G-actin content of urinary cells was 105 units, and in 19 asymptomatic controls was 48.4 units. This difference was significant at $p < 0.001$.

Detailed Description Text (29):

Quantitative Fluorescence Image Analysis of Cells with Multiple Markers

Detailed Description Text (30):

Transitional cell carcinomas (TCC) of the bladder are known to frequently develop as multiple foci in time and place within the bladder. The bladder represents a complex ecosystem of interfacing epithelial and stromal cells, and the progressive subversion of growth and differentiation controls, leading to eventual emergence of cells capable of at least partial autonomous growth, requires years. Altered histopathology is a relatively late event in carcinogenesis, but biochemical or genetic manifestations of carcinogenic damage may be detectable years earlier. As described below, phenotypic markers were mapped in the bladder by quantifying markers in sample specimens from the tumor, the adjacent epithelium and from distant epithelium in persons with bladder cancer and in bladder cells from normal persons. Because quantitative rather than qualitative differences in gene expression and protein levels probably underlay most of the differences between malignant and normal phenotypes, the ability to quantify markers is needed. Accordingly, QFIA was used to quantify the phenotypic markers in single cells from the sample specimens. Because of its visual morphologic component, and in contrast to methods such as flow cytometry, QFIA can link conventional morphologic assessment with quantitative biochemical markers at the single cell level. The markers included; QFIA cytology, a combination of visual morphology and the presence of cells with DNA in excess of 5C (which is a marker for genetic instability), the p300 tumor-related antigen detected by the M344 monoclonal antibody, the differentiation-related proteins epidermal growth factor receptor (EGFR) and G-actin, and p185, a protein product of the HER-2/neu oncogene.

Detailed Description Text (34):

Filters are image analysis quality (DRLP grade) with high precision in angle of incidence to avoid problems with image registration. A/R coated rear surface dichroics eliminate additional undesired stray light for quantitative purposes. Narrow band emission filters are selected to maximize fluorochrome properties and minimize non-specific autofluorescence (see Table IV).

Detailed Description Text (39):

FIGS. 5A-C: Cumulative distributions of EGFR (FIG. 5A), p185 (FIG. 5B) and G-actin (FIG. 5C) as measured by IGL (integrated grey level), Log[IGL] and AGL (average grey level), respectively. IGL and AGL are calculated by the IBAS image analysis system (Zeiss Instruments) from the digitized, grey-level fluorescence images and represent the fluorescence intensity integrated over a cell image (IGL) and the average intensity (AGL) of all pixels comprising the image. Markers were quantified using the IBAS on a cell by cell basis from 50-100 cells per slide. In tumor biopsies, regions of tumor cells were specifically analyzed. In nontumor specimens, cells were randomly selected. In both cases infiltrating lymphocytes or blood cells were specifically excluded. The immunofluorescence assays were calibrated against cell

lines known to express high and low amounts of the proteins. The 18-3-7 line transfected with an expression vector containing HER-2/neu and the A431 line served as positive controls for p185 and EGFR respectively. A large number of slides were prepared from a single batch of each cell line. A positive (with primary antibody) and negative (without primary antibody) slide for each marker was included with each batch of patient samples, and the cells were labeled and analyzed as described. The IGL or AGL was corrected for background fluorescence by subtracting the mean IGL and AGL determined from approximately 100 cells on the negative control slide.

-.quadrature.--control; -.circle-solid.--field of low grade TCC;
-.tangle-solidn.--low grade TCC; -.box-solid.--field of high grade TCC, and
-*--high grade TCC.

Detailed Description Text (49):

These results indicate that the differentiation-related cytoskeletal protein G-actin seems to reflect very early events in bladder carcinogenesis, being abnormal in 60% of the distant biopsies from bladders that contain tumors and essentially 100% of tumors themselves. Previous findings of decreased F-actin in bladder cancer and a strong relation to bladder cancer risk suggested that elevated G-actin (which is the monomeric precursor of F-actin) should be observed. The data presented here confirm the concept that alteration in the cytoskeletal reflected by a shift from a high level of microfilament actin (F-actin) to a high level of globular actin (G-actin) represents an early, common marker for dedifferentiation and shows this phenotypic change persists during cytologic dedifferentiation.

Detailed Description Text (57):

Comparison of Results Between Immunocytochemistry and Quantitative Fluorescence Image Analysis

Detailed Description Text (74):

a. Quantitative Fluorescence Image Analysis. Three markers were simultaneously analyzed. Each sample was centrifuged and the pellet resulting was taken up in buffered fixative and frozen (-70.degree. C.) until analyzed. This procedure preserves marker quantitation and cell morphology. To further minimize the number of inadequate samples, the cells in the sample were counted on a Coulter cell counter using algorithm that takes into account crystals, if present, small cells, such as lymphocytes, and large urothelial cells. This count is used to determine whether the cells will be aliquoted into one container or two. The samples are thawed, diluted with buffer, collected onto a filter and imprinted onto a special slide adapted for use on a Code-On automated stainer/labeler. These methods are described in more detail in the sections "Fixative/Preservative Solution" and "Slide Preparation".

Detailed Description Text (76):

Markers were quantified on individual cells on a cell-by-cell basis for 50-100 cells per slide using a Zeiss IBAS image analysis system equipped for quantitative fluorescence. A schematic diagram of the IBAS system is shown in FIGS. 12A-C (neural net computer was not used in this example). In tumor biopsies, regions of tumor cells were specifically analyzed, but cells were randomly selected in nontumor control specimens. In both cases infiltrating lymphocytes or blood cells were specifically excluded. The images of labeled cells were captured and the intensity of each pixel (dot comprising the image) was converted to a digital grey level between 0 and 255. Immunofluorescence was measured as the integrated grey level (IGL) or averaged grey level (AGL). AGL is the average grey level of the pixels comprising an image and is proportional to the average concentration within a cell. IGL is the value obtained multiplied by the area of the cell image and is proportional to the total content of marker in each cell.

Detailed Description Text (77):

The immunofluorescence assays were calibrated against cell lines known to express high and low amounts of the proteins. A large number of slides were prepared from a single batch of each cell line. A positive (with primary antibody) and negative (without primary antibody) slide for each marker was included with each batch of patient samples, and the cells were labeled and analyzed as described. The IGL or AGL was corrected for extraneous background fluorescence by subtracting the mean IGL or AGL determined from approximately 100 cells on the negative control slide.

Detailed Description Text (94):

Additional fixation to ensure preservation of protein markers can be achieved by first mixing the urine sample with a formaldehyde solution to a final concentration of 0.5% (w/v) and allowing the urine/formaldehyde mixture to stand for a time period, e.g., 15 minutes, prior to addition of the above fixative.

Detailed Description Text (119):

The image analysis system consists of an epi-fluorescence microscope equipped with motorized stage, autofocus mechanism, filter wheel containing various degrees of interference neutral density filters, custom built 4FL excitation/emission filter changer, motorized shutters and objective magnification changer (FIGS. 12A-C). All microscope components are controllable by software from the IBAS console as well as manual controls at the microscope. The software applications developed for this instrument use some common modules i.e. initialization of all motors, scan parameters, case file creation, restoration of incomplete runs, location of first field, automatic threshold selection, DNA scene segmentation, artifact rejection, DNA quantification, image clipping, image review and rejection, and image and data storage. The features selected for measurement, pre-processing of images, scene segmentation, threshold selection, and which constitute features of a cell of a desired type, are determined by the specific marker of interest.

Detailed Description Text (120):

An example of the program flow for a double-label assay, such as for DNA and G-actin is shown in FIGS. 13-14B. Briefly described, all motors are initialized when the instrument is powered up. In this process, home is located and defined for all motors and methods of communication to devices are established where appropriate. The meander pattern desired, optimal neutral density filters and exciters for each marker, and number of desired images of each marker are input by the user. The optimal focus is established in a brief training session for DNA. The cases loaded on the multiple position slide stage are then input into a scroll screen customized for quick entry. This information includes the filenames to be used for data and image storage, laboratory accession numbers to be stored with individual cell measurements, and patients names for each loaded stage position.

Detailed Description Text (121):

Upon completion of data entry, the software takes control and operates unattended until all cases have been completed (FIGS. 14A-B). During this time, selected features are measured and stored in temporary databases while images are clipped and stored to optical disk. The scan on each case continues with movement of the X and Y motors, focus, image capture, artifact rejection, image enhancement of each field for both markers simultaneously until enough cells have been measured (i.e., definitive positive or the entire slide).

Detailed Description Text (122):

The operator is then presented with a gallery of selected cells in order to exclude additional artifacts for each case. Alternatively, the gallery is sent to a neural net computer trained to exclude additional artifacts. The manual operation is controlled by the digitizer with a point and click approach to each image. The final histogram is presented with options to adjust the DNA 2C peak based on internal control cells if desired. Rejection of artifacts for both markers is included with subsequent production of the final report on a laserjet printer. Permanent databases are created and stored in the data path directory. The program then loops back to the case load module allowing additional slides to be analyzed.

Detailed Description Text (123):

In the event that a power failure occurred or the software is terminated while analyzing samples, a module is included to restore incomplete data in which temporary files created during the scans are restored, images reviewed, final reports generated, and permanent databases created.

Detailed Description Text (126):

The triple label software (FIG. 18) for touch preps has been designed to be interactive with the user in selection of cells and fields. It has additional features which allow the user to separate cells in tissue fragments using the digitizer. The three markers from each selected cell can be simultaneously measured

with data and image storage if desired (FIGS. 19A-B). The software allows for image retrieval of stored images or direct image capture of live images from the microscope. Data analysis is also interactive with user selected parameters, scales, and three-dimensional histograms of selected parameters.

Detailed Description Text (127):

Referring in more detail to FIGS. 16A-B, the Rare Event scanning begins at low magnification. A portion of the prepared slide, such as a single field at low power is located and irradiated with a wavelength of light effective in irradiating the dye labeled to DNA. The resulting field image of the microscope field is digitized and analyzed for object images which are images of objects within the criteria established for cells. If the field image is the first which appears to contain cells, a grey value threshold for DNA is calculated. Another review step of the field image is conducted by the microscope wherein object images which exceed a predetermined size threshold or which are smaller than a minimum size threshold are rejected as artifacts.

Detailed Description Text (129):

Object images which survive this review step undergo further analysis. In particular, object images are reviewed more slowly to examine the nuclei. At this stage objects which lack nuclei, such as cell fragments, or have more than one nucleus are rejected. At this stage, cells abnormal for DNA and tissue fragments with DNA are detected and their coordinate locations are recorded. Similarly, the field is irradiated with an excitation wavelength for the M344-conjugated fluorochrome. The resulting field image is corrected for sources of extraneous fluorescence such as background fluorescence and autofluorescence, and for camera shading. The field image is then segmented into discrete object images which are reviewed for a positive appearance for M344 (i.e., fluorescence intensity exceeds a predetermined threshold) and which satisfy desired morphological requirements for size and shape. If positive object images are identified, their coordinate locations are recorded. Images of these selected cells may be stored. Positive object images are searched for until the entire slide has been examined or until a predetermined minimum number of recorded M344 positive images has been exceeded. An example of such a predetermined threshold is 20 positive images per 5,000 cells on the prepared slide.

Detailed Description Text (130):

Once the field image has been searched under the first magnification (low power e.g. 12.5.times. power for DNA, 25.times. for most other markers), each object image identified as abnormal image is viewed again under a second, higher, magnification (e.g. 25.times. power for DNA) (FIG. 17) during which the high power object image is reviewed again for comparison with predetermined selection requirements and DNA is quantified. For example, objects showing cells with nuclei touching or other cell fragments are eliminated from consideration. Object images which pass this selection process are reviewed again more slowly and are compared to selection parameters related to cell shape. Additionally, DNA is quantified at this point. If the object images survive this review, and are still considered to be morphologically abnormal, or have DNA in excess of a predetermined amount (e.g., $\geq 5C$), object images are stored.

Detailed Description Text (131):

The slide is searched for positive object images until the entire slide has been examined or until a predetermined number of cells with abnormal DNA has been recorded (e.g. until the gallery is full of stored images). Under high magnification, a random scan for cells with abnormal DNA continues until at least 100 cells are measured. In the case of G-actin, the scan continues until 100 cells have been measured for G-actin or until the DNA scan is complete.

Detailed Description Text (132):

The stored object images of abnormal cells, cells with abnormal amounts of DNA or cells positive for M344 (or positive for a similar marker, or having particular quantities thereof) may be reviewed again by another method for confirmation. For example, the object images may be confirmed by a trained operator, or the stored object images may be delivered to an automated confirmation system such as a neural net computer trained with a library of normal, abnormal, positive, negative and

false positive cell images.

Detailed Description Text (137):

In the present invention, the preferred method for accounting for error due to autofluorescence is to measure the autofluorescent light emitted from each cell upon excitation by a predetermined excitation wavelength. The grey level image of the cell is then corrected on a pixel-by-pixel basis.

Detailed Description Text (139):

This could be done by calculating an average autofluorescence over the entire image, then subtracting the same average amount of autofluorescence from each pixel of the image. However, the autofluorescent component can vary from pixel to pixel. Therefore, a method which subtracts only an average autofluorescent value can still result in significant error in any given pixel. To determine the amount of autofluorescence for a given pixel a second excitation wavelength is chosen from the tail of the fluorochrome's excitation spectrum. This wavelength is significantly different from the peak excitation wavelength of the fluorochrome (FIG. 20).

Detailed Description Text (140):

When the slide is irradiated with this second autofluorescence wavelength, most of the fluorescent light which is emitted is autofluorescence, with a small portion being emitted from the fluorochrome. In effect, the autofluorescence wavelength mildly excites the fluorochrome but does not cause a high level of excitation such as is caused by the peak excitation wavelength for the fluorochrome. The fluorescence emission is then digitized for each pixel in the image. This fluorescence is mostly autofluorescence.

Detailed Description Text (141):

Each calculated autofluorescence grey level is subtracted from the grey level obtained for that pixel when the slide is irradiated with the excitation wavelength of the fluorochrome. The resulting grey level value is the pixel grey level corrected for autofluorescence and primarily represents the quantity of the fluorochrome in that portion of the cell. Most of the autofluorescence is effectively removed from the image. This correction is made for each pixel of each cell measured.

Detailed Description Text (163):

This example relates to directly encoding gray level images for classification by neural nets for use in automated cancer diagnosis or in detection of abnormal cells resulting from the process of carcinogenesis.

Detailed Description Text (165):

The neural network is a device for "learning." If presented with a set of complex patterns that are classified by a human observer, the neural network can "learn" to recognize members of each of the classes it was "trained" to recognize. In addition, if new objects that the neural net has not been trained to recognize are presented, the neural network is capable of determining that such objects do not fit any current classification. Neural networks are, in general, tolerant of some variation in characteristics within a given class. Neural networks are also rapid, usually achieving at least a near real time performance, even with complex images.

Detailed Description Text (166):

Though the concept of using an artificial neural network system (ANNS) to solve pattern recognition has been proposed since the fifties, recent advancement of learning theory and adaptive signal processing has greatly increased and strengthened the use of ANNS for practical problems. Research projects directed to applications of artificial neural networks can be roughly categorized into three main areas: pattern recognition/associative memory, artificial intelligence, and optimization. Pattern recognition problems have direct applications in robotics, machine vision, and natural language understanding. Artificial intelligence problems include game theory, and other heuristically oriented applications. Optimization problems include modelling, estimation, prediction, and control. One application of neural networks is in recognition of visual images, such as is required in the present invention.

Detailed Description Text (168):

In the present invention, neural networks are used as part of an automated system wherein encoded grey level images are utilized in cancer diagnosis and cancer risk assessment.

Detailed Description Text (174):

In this example, an image analysis system was used to perform preliminary algorithmic classification of images of exfoliated urine cells stained with a fluorescent dye which preferentially labels DNA and then to capture the grey-level images of potentially abnormal cells for analysis by a neural network. Digitized, grey-level cell images were captured on disks and were minimally processed for analysis by a trained neural network implemented on a Prime computer (VAX 780 equivalent). Minimally processed means normalized such that differences in DNA content were not a factor in the decision. The network consisted of an input layer (1936 neurodes), two hidden layers (50 and 24 neurodes each) and a single output node.

Detailed Description Text (175):

The network was trained with an image set consisting of both low and high grade cancer cells and several different examples of noncancer cells. A second, test set was evaluated without disagreement with a human expert (Table XVI). The principle was proven unequivocally that grey level images of cells having DNA labeled with fluorescent probes could serve as direct input for a neural network and that such networks could be trained to differentiate cancer cells from noncancer cells. The results clearly demonstrated the feasibility of using neural networks to recognize and classify grey level images captured by an image analysis microscope.

Detailed Description Text (176):

In a system for evaluating more than one marker, an image library for each marker would be selected. This is based upon the assumption that markers do not interfere with each other, an assumption that is satisfied using the processing techniques described herein for the present invention.

Detailed Description Text (178):

Image library: An image library was collected by an expert cytologist with a Zeiss IBAS system. The IBAS is a full-function image analysis system with image capture capabilities. The cells were labeled with H-33258, a fluorescent dye that labels DNA preferentially. The images were stored as 512.times.512 pixel gray level images on floppy disks and sent for preliminary image processing to extract the image of the cell in a 64.times.64 format. These images were then analyzed at another site. An adequate image library is selected from cell images from patients known to have certain clinical conditions. Thus, cells representing cancer are taken from cells found in the urine of patients known to have cancer. Cells representing the normal condition are taken from individuals having no indications of cancer. Cells representing premalignant conditions are taken from patents who previously had a cancer but who do not currently have a detectable cancer, and who have one or more abnormal quantitative markers. Cells representing false results are obtained from patients being seen for urologic conditions other than cancer (e.g., chronic infection or benign prostatic hyperplasia) who have been evaluated and found to be free of cancer. The cells are then characterized by an expert cytologist (e.g., normal cell). Thus, each image will have associated two characteristics, its intrinsic classification and the clinical condition of the patient. An example might be "normal cell from cancer patient."

Detailed Description Text (179):

The image library consisted of the following cell types:

Detailed Description Text (180):

Normal cells from noncancer urines: Squamous cells (12 images). These are often found in female urine and represent cells that are differentiating along a more skin cell-like pathway. Transitional urothelial cells (12 images) represent the usual cells lining the bladder. Cancers are generally derived from this cell type. Polymorphonuclear leukocytes PML (8 images) are white cells found in the urine as a response to infection or inflammation.

Detailed Description Text (181):

Abnormal cells from cancer cases: Mild-moderate atypical cells (12 images) express mild morphological changes. These can be derived from both low-grade cancers and noncancer causes, and the challenge of bladder cancer diagnosis is to identify those that are derived from cancer. Moderately-severely atypical cells (12 images) are more severely altered. Suspicious cells (16 images) are found in high-grade tumors and have a characteristic abnormal appearance.

Detailed Description Text (182):

A selected image library was prepared as follows. Normal: Squamous cells (10 images), Transitional cells (2 images), PML (1 image); Cancer cases: Suspicious cells (10 images).

Detailed Description Text (185):

The training time was approximately 24 hours. The time to classify a test image, however, was only 2 minutes of calculation time. Table XVI demonstrates the concordance between the human expert and the trained neural network with the second, independent test set. All test cell images were correctly classified as either normal cell or abnormal cell. It is of interest that this was achieved with a training set that emphasized squamous cells over transitional cells, while the test set was richer in transitional cells. This demonstrates that the results achieved with the neural net may have a degree of generalizability.

Detailed Description Text (186):

A truly improved hybrid system will use algorithmic processing to identify potentially abnormal objects and a neural network to at least partially replace the human in classification of cell images. Hardware implementations of neural networks are commercially available. Hardware implementation will permit the true massive parallel processing rather than software simulation in a linear sequentially-processing digital computer. The approximate 10 μ s required to process an image with hardware implementation will make classification of each image feasible. A reduction in training times can be achieved by using a more powerful computer, by various image-compression techniques to reduce the number of elements in the image, or by a combination of approaches.

Detailed Description Text (189):

Described herein is a new multi-threshold modified Perceptron capable of handling both binary and analog input. The modified Perceptron replaces the sigmoid function with sinusoidal function. A computer program was developed to simulate behavior of a network utilizing the modified Perceptron. A network utilizing this modified Perceptron requires fewer number of iterations to converge to a solution than that of a multi-layer Perceptron network using back propagation. A hybrid multi-layer network using the modified and sigmoidal perceptron was used to classify images of bladder cells. The results indicated that the hybrid network was capable of correctly classifying the images.

Detailed Description Text (208):

The modified perceptron was combined with the traditional perceptron to form a more powerful multi-layer network to analyze images labelled with the M344 antibody. This network consisted of an input layer, an output layer and one hidden layer. The hidden layer and the output layer neurons used the sinusoidal and sigmoid nonlinearity functions, respectively. The network was trained using two different sets of training data obtained from the recorded images. The recorded images were up to 70.times.70 pixels.

Detailed Description Text (209):

The first approach utilized a 10.times.10 set of pixels from the center of each cell. Therefore, the network required 100 input neurons. Hidden layer had 10 sinusoidal neurons and the output layer had one sigmoidal neuron. A total of 60 training sets was used to train the network to distinguish between cells falsely positive for the M344 marker and negative cells. A total of four test cases were used. The four represented two positive cells, one negative cell, and one false positive cell. The network required an average of 195 iterations to learn the data. The average number of iterations (maximum of 870 and minimum of 57 iterations) corresponds to different seed numbers used for the random number generator. The

network successfully classified the four test cases. This approach was not able to correctly learn four of the images used for the training set.

Detailed Description Text (210):

The second approach used 55 images for training and 8 for testing the network. The test set consisted of 4 positive, 2 negative, and 2 false positive. Each image was reduced to 60.times.60 pixels.

Other Reference Publication (2):

G. P. Hemstreet, et al., "Quantitative Fluorescence Image Analysis in Bladder Cancer Screening", Journal of Occupational Medicine, vol. 32, No. 9, Sep. 1990, pp. 822-828.

Other Reference Publication (4):

R. A. Bass, et al., "DNA Cytometry and Cytology By Quantitative Fluorescence Image Analysis in Symptomatic Bladder Cancer Patients", International Journal Cancer, 40, 1987, pp. 698-705.

Other Reference Publication (9):

W. L. Parry, et al., "Cancer Detection By Quantitative Fluorescence Image Analysis", The Journal of Urology, vol. 139, Feb. 1988, pp. 270-274.

Other Reference Publication (13):

G. P. Hemstreet, et al., "Identification of a High Risk Subgroup of Grade 1 Transitional Cell Carcinoma Using Image Analysis Based Deoxyribonucleic Acid Ploidy Analysis of Tumor Tissue", The Journal of Urology, vol. 146, Dec. 1991, pp. 1525-1529.

CLAIMS:

1. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide, the portion of the cell sample treated with a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the cell sample prior to application of the portion of the cell sample to the slide leaving the prepared slide substantially free of crystals for improving microscopic analysis of the cell on the prepared slide, then treating the slide with a fluorescent label for labeling the cytological marker to form a labeled cytological marker;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in a cell to emit fluorescent light having an emission wavelength for forming a field image;

using a microscope means to select cell images on the field image;

obtaining a number related to the selected cell images;

and

outputting the number for use in classifying the cell sample.

2. The method of claim 1 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

7. The method of claim 1 wherein the step of using a microscope means is preceded by the step of correcting the field image for autofluorescence.

8. The method of claim 1 wherein the step of obtaining a number is preceded by the step of reviewing the selected cell images with a confirmation means confirming that the selected cell images represent cells of a desired type.

9. The method of claim 8 wherein in the step of reviewing the selected cell images the confirmation means is a neural net computing means.

12. The method of claim 11 further comprising the additional step of analyzing the prepared slide for the second cytological marker comprising:

irradiating a second portion of the prepared slide with an amount of a second excitation wavelength of light effective in causing the cells containing the second fluorescent label to emit fluorescent light having a second emission wavelength for forming a second field image wherein the second portion may be the same as the first portion,

using the microscope means to select second cell images on the second field image, obtaining a second number related to the selected second cell images, and outputting the second number for use in classifying the cell sample.

13. The method of claim 12 wherein the step of using the microscope means to select second cell images is preceded by the step of correcting the second field image for autofluorescence.

14. The method of claim 12 wherein the step of obtaining a second number is preceded by the step of reviewing the selected second cell images with a confirmation means for confirming that the selected second cell images represent cells of a desired type.

15. The method of claim 14 wherein in the step of reviewing the selected second cell images the confirmation means is a neural net computing means.

18. The method of claim 17 further comprising the additional step of analyzing the prepared slide for the third cytological marker comprising:

irradiating a third portion of the prepared slide with an amount of a third excitation wavelength of light effective in causing the cells containing the third fluorescent label to emit fluorescent light having a third emission wavelength for forming a third field image, wherein the third portion may be the same as the second portion or the first portion,

using the microscope means to select third cell images on the field third image, obtaining a third number related to the selected third cell images, and outputting the third number for use in classifying the cell sample.

19. The method of claim 18 wherein the step of using the microscope means to select third cell images is preceded by the step of correcting the third field image for autofluorescence.

20. The method of claim 18 wherein the step of obtaining a third number is preceded by the step of reviewing the selected third cell images with a confirmation means for confirming that the selected third cell images represent cells of a desired type.

21. The method of claim 20 wherein in the step of reviewing the selected third cell images the confirmation means is a neural net computing means.

23. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide, the portion of the cell sample treated with a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the cell sample prior to application of the portion of the cell sample to the slide leaving the

prepared slide substantially free of crystals for improving microscopic analysis of the cell on the prepared slide, then treating the slide with a first fluorescent label for labeling the first cytological marker to form a labeled first cytological marker and the second fluorescent label for labeling the second cytological marker to form a labeled second cytological marker;

irradiating a first portion of the prepared slide with an amount of a first excitation wavelength of light effective in causing the first fluorescent label in the cell to emit fluorescent light having a first emission wavelength for forming a first field image;

using a microscope means to select first cell images on the first field image;

obtaining a first number related to the selected first cell images;

irradiating the second portion of the prepared slide with a second excitation wavelength of light effective in causing the second fluorescent label to emit fluorescent light having a second emission wavelength for forming a second field image wherein the second portion may be the same as the first portion;

using the microscope means to select second cell images on the second field image;

obtaining a second number related to the selected second cell images; and

outputting the first number and the second number for use in classifying the cell sample.

24. The method of claim 23 wherein the step of using a microscope means to select first cell images is preceded by the step of correcting the first field image for autofluorescence.

25. The method of claim 23 wherein the step of obtaining a first number is preceded by the step of reviewing the selected first cell images with a confirmation means for confirming that the selected cell images represent cells of a desired type.

26. The method of claim 25 wherein in the step of reviewing the selected first cell images the confirmation means is a neural net computing means.

27. The method of claim 23 wherein the step of obtaining a second number is preceded by the step of reviewing the selected second cell images with a confirmation means for confirming that the selected second cell images represent cells of a desired type.

28. The method of claim 27 wherein in the step of reviewing the selected second cell images the confirmation means is a neural net computing means.

29. The method of claim 23 wherein in the step of providing a prepared slide the first cytological marker and the second cytological marker are selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

30. The method of claims 23 wherein in the step of providing a prepared slide the first fluorescent label and the second fluorescent label are selected from the group consisting of Hoechst 33258, M344 plus a fluorescent conjugate, an anti-HER-2/neu protein probe plus a fluorescent conjugate, an anti-EGFR probe plus a fluorescent conjugate, and DNase I plus a fluorescent conjugate.

32. The method of claim 23 wherein the first cytological marker is DNA and the second cytological marker is the p300 protein antigen.

36. The method of claim 35 further comprising the additional step of analyzing the prepared slide for the third cytological marker comprising:

irradiating a third portion of the prepared slide with an amount of a third excitation wavelength of light effective in causing the cells containing the third fluorescent label to emit fluorescent light having a third emission wavelength for

forming a third field image wherein the third portion may be the same as the second portion or the first portion,

using the microscope means to select third cell images on the third field image,
obtaining a third number related to the selected third cell images, and
outputting the third number for use in classifying the cell sample.

37. The method of claim 36 wherein the step of using the microscope means to select third cell images is preceded by the step of correcting the third field image for autofluorescence.

38. The method of claim 36 wherein the step of obtaining a third number is preceded by the step of reviewing the selected third cell images with a confirmation means for confirming that the selected third cell images represent cells of a desired type.

39. The method of claim 38 wherein in the step of reviewing the selected third cell images the confirmation means is a neural net computing means.

41. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide, the portion of the cell sample treated with a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the cell sample prior to application of the portion of the cell sample to the slide leaving the prepared slide substantially free of crystals for improving microscopic analysis of the cell on the prepared slide, then treating the slide with a first fluorescent label for labeling the first cytological marker to form a labeled first cytological marker, a second fluorescent label for labeling the second cytological marker to form a labeled second cytological marker, and a third fluorescent label for labeling the third cytological marker to form a labeled third cytological marker;

irradiating a first portion of the prepared slide with an amount of a first excitation wavelength of light effective in causing the first fluorescent label in the cell to emit fluorescent light having a first emission wavelength for forming a first field image;

using a microscope means to select first cell images on the first field image;

obtaining a first number related to the selected first cell images;

irradiating a second portion of the prepared slide with a second excitation wavelength of light effective in causing the second fluorescent label to emit fluorescent light having a second emission wavelength for forming a second field image visible wherein the second portion may be the same as the first portion;

using the microscope means to select second cell images on the second field image;

obtaining a second number related to the selected second cell images;

irradiating a third portion of the prepared slide with a third excitation wavelength of light effective in causing the third fluorescent label to emit fluorescent light having a third emission wavelength for forming a third field image wherein the third portion may be the same as the second portion or the first portion;

using the microscope means to select third cell images on the third field image;

obtaining a third number related to the selected third cell images; and

outputting the first number, the second number, and the third number for use in classifying the cell sample.

42. The method of claim 41 wherein the step of using a microscope means to select first cell images is preceded by the step of correcting the first field image for autofluorescence.

43. The method of claim 41 wherein the step of obtaining a first number is preceded by the step of reviewing the selected first cell images with a confirmation means for confirming that the selected cell images represent cells of a desired type.

44. The method of claim 43 wherein in the step of reviewing the selected first cell images the confirmation means is a neural net computing means.

45. The method of claim 41 wherein the step of using the microscope means to select second cell images is preceded by the step of correcting the second field image for autofluorescence.

46. The method of claim 41 wherein the step of obtaining a second number is preceded by the step of reviewing the selected second cell images with a confirmation means for confirming that the selected second cell images represent cells of a desired type.

47. The method of claim 46 wherein in the step of reviewing the selected second cell images the confirmation means is a neural net computing means.

48. The method of claim 41 wherein the step of using the microscope means to select third cell images is preceded by the step of correcting the third field image for autofluorescence.

49. The method of claim 41 wherein the step of obtaining a third number is preceded by the step of reviewing the selected third cell images with a confirmation means for confirming that the selected third cell images represent cells of a desired type.

50. The method of claim 49 wherein in the step of reviewing the selected third cell images the confirmation means is a neural net computing means.

51. The method of claim 41 wherein in the step of providing a prepared slide the first cytological marker, the second cytological marker and the third cytological marker are selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

52. The method of claim 41 wherein in the step of providing a prepared slide the first fluorescent label, the second fluorescent label and the third fluorescent label are selected from the group consisting of Hoechst 33258, M344 plus a fluorescent conjugate, an anti-HER-2/neu protein probe plus a fluorescent conjugate, an anti-EGFR probe plus a fluorescent conjugate, and DNase I plus a fluorescent conjugate.

54. The method of claim 41 wherein in the step of providing a prepared slide the first cytological marker is DNA and the second cytological marker is selected from the group consisting of p300, actin, EGFR and HER-2/neu protein.

55. The method of claim 41 wherein in the step of providing a prepared slide the first cytological marker is DNA, the second cytological marker is the p300 protein antigen and the third cytological marker is selected from the group consisting of EGFR, actin and HER-2/neu protein.

56. The method of claim 41 wherein in the step of providing a prepared slide the first cytological marker is DNA, the second cytological marker is actin and the third cytological marker is selected from the group consisting of p300, EGFR and HER-2/neu protein.

58. A method of analyzing a cell sample, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a

portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

digitizing the field image;

converting the field image into a plurality of digitized pixels;

obtaining for each pixel of the field image an intensity measurement of the peak emission wavelength;

correcting the field image for autofluorescence by selecting an off peak emission wavelength, obtaining an intensity measurement of the off peak emission wavelength for each pixel of the field image, and subtracting the intensity measurement of the off peak emission wavelength from the intensity measurement of the peak emission wavelength for each pixel of the field image wherein is obtained a corrected field image;

using a microscope means to select cell images on the corrected field image;

obtaining a number related to the selected cell images; and

outputting the number for use in classifying the cell sample.

59. The method of claim 58 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

64. The method of claim 58 wherein the step of obtaining a number is preceded by the step of reviewing the selected cell images with a confirmation means confirming that the selected cell images represent cells of a desired type.

65. The method of claim 64 wherein in the step of reviewing the selected cell images the confirmation means is a neural net computing means.

68. A method of analyzing a cell sample, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a first fluorescent label for labeling a first cytological marker to form a labeled first cytological marker and a second fluorescent label for labeling a second cytological marker to form a labeled second cytological marker;

irradiating a first portion of the prepared slide with an amount of a first excitation wavelength of light effective in causing the first fluorescent label in the cell to emit fluorescent light having a first peak emission wavelength for forming a first field image;

converting the first field image into a plurality of digitized pixels;

obtaining for each pixel of the first field image an intensity measurement of the first peak emission wavelength;

correcting the first field image for autofluorescence by selecting a first off peak emission wavelength, obtaining an intensity measurement of the first off peak emission wavelength for each pixel of the first field image, and subtracting the intensity measurement of the first off peak emission wavelength from the intensity measurement of the first peak emission wavelength for each pixel of the first field image wherein is obtained a corrected first field image;

using a microscope means to select first cell images on the corrected first field image;

obtaining a first number related to the selected first cell images;

irradiating a second portion of the prepared slide with a second excitation wavelength of light effective in causing the second fluorescent label to emit fluorescent light having a second emission wavelength for forming a second field image wherein the second portion may be the same as the first portion;

converting the second field image into a plurality of digitized pixels;

obtaining for each pixel an intensity measurement of the second peak emission wavelength;

correcting the second field image for autofluorescence by selecting a second off peak emission wavelength, obtaining an intensity measurement of the second off peak emission wavelength for each pixel of the second field image, and subtracting the intensity measurement of the second off peak emission wavelength from the intensity measurement of the second peak emission wavelength for each pixel of the second field image wherein is obtained a corrected second field image;

using the microscope means to select second cell images on the corrected second field image;

obtaining a second number related to the selected second cell images; and

outputting the first number and the second number for use in classifying the cell sample.

71. The method of claim 68 wherein the step of obtaining a first number is preceded by the step of reviewing the selected first cell images with a confirmation means for confirming that the selected cell images represent cells of a desired type.

72. The method of claim 71 wherein in the step of reviewing the selected first cell images the confirmation means is a neural net computing means.

73. The method of claim 68 wherein the step of obtaining a second number is preceded by the step of reviewing the selected second cell images with a confirmation means for confirming that the selected second cell images represent cells of a desired type.

74. The method of claim 73 wherein in the step of reviewing the selected second cell images the confirmation means is a neural net computing means.

75. The method of claim 68 wherein in the step of providing a prepared slide the first cytological marker and the second cytological marker are selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

76. The method of claim 68 wherein in the step of providing a prepared slide the first fluorescent label and the second fluorescent label are selected from the group consisting of Hoechst 33258, M344 plus a fluorescent conjugate, an anti-HER-2/neu protein probe plus a fluorescent conjugate, an anti-EGFR probe plus a fluorescent conjugate, and DNase I plus a fluorescent conjugate.

78. The method of claim 68 wherein the first cytological marker is DNA and the second cytological marker is the p300 protein antigen.

81. A method of analyzing a cell sample, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a first fluorescent label for labeling a first cytological marker to form a labeled first cytological marker, a second fluorescent label for labeling a second cytological marker to form a labeled second cytological marker, and a third fluorescent label for labeling a third cytological marker to form a labeled third cytological marker;

irradiating a first portion of the prepared slide with an amount of a first excitation wavelength of light effective in causing the first fluorescent label in the cell to emit fluorescent light having a first peak emission wavelength for forming a first field image;

converting the first field image into a plurality of digitized pixels;

obtaining for each pixel of the first field image an intensity measurement of the first peak emission wavelength;

correcting the first field image for autofluorescence by selecting a first off peak emission wavelength, obtaining an intensity measurement of the first off peak emission wavelength for each pixel of the first field image, and subtracting the intensity measurement of the first off peak emission wavelength from the intensity measurement of the first peak emission wavelength for each pixel of the first field image wherein is obtained a corrected first field image;

using a microscope means to select first cell images on the corrected first field image;

obtaining a first number related to the selected first cell images;

irradiating a second portion of the prepared slide with a second excitation wavelength of light effective in causing the second fluorescent label to emit fluorescent light having a second emission wavelength for forming a second field image wherein the second portion may be the same as the first portion;

converting the second field image into a plurality of digitized pixels;

obtaining for each pixel an intensity measurement of the second peak emission wavelength;

correcting the second field image for autofluorescence by selecting a second off peak emission wavelength, obtaining an intensity measurement of the second off peak emission wavelength for each pixel of the second field image, and subtracting the intensity measurement of the second off peak emission wavelength from the intensity measurement of the second peak emission wavelength for each pixel of the second field image wherein is obtained a corrected second field image;

using the microscope means to select second cell images on the corrected second field image;

obtaining a second number related to the selected second cell images;

irradiating a third portion of the prepared slide with a third excitation wavelength of light effective in causing the third fluorescent label to emit fluorescent light having a third peak emission wavelength for forming a third field image wherein the third portion may be the same as the second portion or the first portion;

correcting the third field image for autofluorescence by selecting a third off peak emission wavelength, obtaining an intensity measurement of the third off peak emission wavelength for each pixel of the third field image, and subtracting the intensity measurement of the third off peak emission wavelength from the intensity measurement of the third peak emission wavelength for each pixel of the third field image wherein is obtained a corrected third field image;

using the microscope means to select third cell images on the corrected third field image;

obtaining a third number related to the selected third cell images; and

outputting the first number, the second number, and the third number for use in classifying the cell sample.

82. The method of claim 81 wherein the step of obtaining a first number is preceded

by the step of reviewing the selected first cell images with a confirmation means for confirming that the selected first cell images represent cells of a desired type.

83. The method of claim 82 wherein in the step of reviewing the selected first cell images the confirmation means is a neural net computing means.

84. The method of claim 81 wherein the step of obtaining a second number is preceded by the step of reviewing the selected second cell images with a confirmation means for confirming that the selected second cell images represent cells of a desired type.

85. The method of claim 84 wherein in the step of reviewing the selected second cell images the confirmation means is a neural net computing means.

86. The method of claim 81 wherein the step of obtaining a third number is preceded by the step of reviewing the selected third cell images with a confirmation means for confirming that the selected third cell images represent cells of a desired type.

87. The method of claim 81 wherein in the step of reviewing the selected third cell images the confirmation means is a neural net computing means.

89. The method of claim 81 wherein in the step of providing a prepared slide the first cytological marker, the second cytological marker and the third cytological marker are selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

90. The method of claim 81 wherein in the step of providing a prepared slide the first fluorescent label, the second fluorescent and the third fluorescent label are selected from the group consisting of Hoechst 33258, M344 plus a fluorescent conjugate, an anti-HER-2/neu protein probe plus a fluorescent conjugate, an anti-EGFR probe plus a fluorescent conjugate, and DNase I plus a fluorescent conjugate.

92. The method of claim 81 wherein in the step of providing a prepared slide the first cytological marker is DNA and the second cytological marker is selected from the group consisting of p300, actin, EGFR and HER-2/neu protein.

93. The method of claim 81 wherein in the step of providing a prepared slide the first cytological marker is DNA, the second cytological is the p300 protein antigen and the third cytological marker is selected from the group consisting of EGFR, actin and HER-2/neu protein.

94. The method of claim 81 wherein in the step of providing a prepared slide the first cytological marker is DNA, the second cytological marker is actin and the third cytological marker is selected from the group consisting of p300, EGFR and HER-2/neu protein.

97. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker and wherein the portion of the cell sample used in preparing the slide was fixated using a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the sample;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

using a microscope means to select cell images on the field image;

reviewing the selected cell images with a neural net computing means for confirming that the selected cell images represent cells of a desired type;

obtaining a number related to the selected cell images; and

outputting the number for use in classifying the cell sample.

98. The method of claim 97 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

103. The method of claim 97 wherein the step of using a microscope means is preceded by the step of correcting the field image for autofluorescence.

105. A method of analyzing a cell sample, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having a peak emission wavelength for forming a field image;

converting the field image into a plurality of digitized pixels;

obtaining for each pixel of the field image an intensity measurement of the peak emission wavelength;

correcting the field image for autofluorescence by selecting an off peak emission wavelength, obtaining an intensity measurement of the off peak emission wavelength for each pixel of the field image, and subtracting the intensity measurement of the off peak emission wavelength from the intensity measurement of the peak emission wavelength for each pixel of the field image wherein is obtained a corrected field image;

using a microscope means to select cell images on the corrected field image;

classifying a cell image as positive or negative for a predetermined quantity of the fluorescent label;

using a neural net computing means to further classify a positive cell image as a true-positive cell image or as a false-positive cell image;

obtaining a parameter related to the number of true-positive cell images; and

outputting the parameter for use in classifying the cell sample.

106. The method of claim 105 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

114. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker and wherein the portion of the cell sample used in preparing the slide was fixated using a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting the formation of substantially all of the crystals in the sample;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

correcting the field image for autofluorescence;

using a microscope means to select cell images on the field image;

obtaining a number related to the selected cell images; and

outputting the number for use in classifying the cell sample.

115. The method of claim 114 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

119. The method of claim 114 wherein the step of obtaining a number is preceded by the step of reviewing the selected cell images with a confirmation means confirming that the selected cell images represent cells of a desired type.

120. The method of claim 119 wherein in the step of reviewing the selected cell images the confirmation means is a neural net computing means.

121. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker and wherein the portion of the cell sample used in preparing the slide was fixated using a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting the formation of substantially all of the crystals in the sample;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

correcting the field image for autofluorescence;

using a microscope means to select cell images on the field image;

reviewing the selected cell images with a neural net computing means for confirming that the selected cell images represent cells of a desired type;

obtaining a number related to the selected cell images; and

outputting the number for use in classifying the cell sample.

122. The method of claim 121 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

127. A method of assessing an individual's risk for bladder cancer, comprising:

providing a prepared slide having a population of cells affixed thereto, the prepared slide having been prepared by applying a portion of a cell sample provided by the individual to a slide then treating the slide with at least a first fluorescent label for labeling cellular DNA and at least a second fluorescent label for labeling cellular p300 protein;

irradiating the prepared slide with an amount of a first excitation wavelength of light effective in causing the first fluorescent label in the cells on the prepared slide to emit fluorescent light having a first emission wavelength;

obtaining a first population parameter related to the number of cells in the population of cells having quantities of cellular DNA which exceed a predetermined threshold quantity of cellular DNA;

irradiating the prepared slide with a second excitation wavelength of light effective in causing the second fluorescent label in the cells on the prepared slide to emit fluorescent light having a second emission wavelength;

obtaining a second population parameter related to the number of cells in the population of cells having quantities of p300 protein which exceed a predetermined threshold quantity of p300 protein;

comparing the first population parameter to a set of predetermined first parameter thresholds;

comparing the second population parameter to a set of predetermined second parameter thresholds; and

assigning a predetermined risk for bladder cancer to the individual based on which first parameter thresholds and second parameter thresholds are exceeded.

132. A method of analyzing a cell sample derived from urine or from a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker and wherein the portion of the cell sample used in preparing the slide was fixated using a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the sample;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

using a microscope means to select cell images on the field image;

classifying the cell image as positive or negative for the fluorescent label;

using a neural net computing means to further classify a positive cell image as a true-positive cell image or as a false-positive cell image;

obtaining a parameter related to the number of true-positive cell images; and

outputting the parameter for use in classifying the cell sample.

133. The method of claim 132 wherein the step of using a microscope means to select cell images is preceded by the step of correcting the field image for autofluorescence.

134. The method of claim 132 wherein the step of classifying the cell image is preceded by the step of reviewing the selected cell images with a confirmation means for confirming that the selected cell images represent cells of a desired type.

135. The method of claim 132 wherein in the step of providing a prepared slide the cytological marker is selected from the group consisting of DNA, p300, actin, EGFR and HER-2/neu protein.

136. The method of claim 135 wherein the cytological marker is the p300 protein antigen.

138. The method of claim 132 wherein the step of providing a prepared slide the fluorescent label is selected from the group consisting of Hoechst 33258, M344 plus a fluorescent conjugate, an anti-HER2/neu protein probe plus a fluorescent conjugate, an anti-EGFR probe plus a fluorescent conjugate, and DNase I plus a fluorescent conjugate.

143. A method of analyzing a cell sample derived from urine or a bladder wash, comprising:

providing a prepared slide, the prepared slide having been prepared by applying a portion of a cell sample to a slide then treating the slide with a fluorescent label for labeling a cytological marker to form a labeled cytological marker and wherein the portion of the cell sample used in preparing the slide was fixated using a fixative composition comprising a salt of ethylenediaminetetraacetic acid effective in inhibiting formation of substantially all of the crystals in the sample;

irradiating a portion of the prepared slide with an amount of an excitation wavelength of light effective in causing the fluorescent label in the cell to emit fluorescent light having an emission wavelength for forming a field image;

using a microscope means to select cell images on the field image;

classifying the cell image as positive or negative for the fluorescent label;

using a neural net computing means to further classify a positive cell image as a true-positive cell image or as a false-positive cell image, wherein the neural net computing means has been previously trained with a training set comprising:

a true-positive image set comprising positive cell images derived from one or more subjects diagnosed as having cancer, and

a false-positive image set comprising positive cell images derived from one or more subjects diagnosed as free of cancer;

obtaining a parameter related to the number of true-positive cell images; and

outputting the parameter for use in classifying the cell sample.

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**** See image for Certificate of Correction ****TITLE: Method and apparatus for determining the amount of oncogene protein product in a cell sampleAbstract Text (1):

An apparatus and method for determining an amount of oncogene protein product copies in a cell includes an optical conversion module for measuring an amount of optically enhanced DNA in a cell sample. A subsystem for measuring an amount of an optically enhanced oncogene protein product protein product is coupled to the DNA measuring means. A subsystem for comparing the measured DNA amount and measured oncogene protein product protein product amount produces a oncogene protein product copy measurement which is fed to an output device for producing an output indicative of the amounts of the oncogene protein product in the cells of the cell sample.

Brief Summary Text (2):

The invention relates to a system for performing an assay of a cell sample to provide an accurate quantitative analysis of a characteristic of the cells which have been sampled. More particularly, the invention is directed to a system which receives images of stained cells and enhances the cell images prior to further processing to determine an amount of oncogene protein product in the cells of a cell sample.

Brief Summary Text (4):

Attempts have been made to automate the cellular examination process. In U.S. Pat. No. 4,741,043 to Bacus for Method and Apparatus for Image Analyses of Biological Specimens, an automated method and a system for measuring the DNA of cells are disclosed which employ differential staining of the DNA in cell nuclei with a Feulgen Azure A stain and image processing.

Brief Summary Text (5):

U.S. application Ser. No. 315,289, filed Feb. 24, 1989, now U.S. Pat. No. 5,086,476 for Method and Apparatus for Determining a Proliferation Index of a Cell Sample to Bacus, assigned to the instant assignee, discloses a system for determining the proliferation index of cells by microscopic examination of cell samples which have been stained with a proliferation substance stain and a nuclear stain. The system includes a computer coupled to a pair of monochrome television cameras, which receive optically filtered images of the magnified cell images, and an image processor. The system computes the proliferation index from the optical characteristics of the stained cell sample.

Brief Summary Text (6):

Recently certain genes have been discovered that appear to contribute to the onset and growth of cancers. These genes, known as oncogenes and proto-oncogenes, also may contribute to the growth and development of human beings in the early stages of their lives. Ongoing research has found that certain of these oncogenes seem to be related to specific cancers. One of them, the neu HER-2 proto-oncogene, appears to be related to human breast and ovarian cancers. It has also recently been found that neu HER-2 proto-oncogenes and the oncogene protein product that is expressed from neu HER-2 appear, when in elevated amounts, to be correlated with the virulence of the cancer, Slamon D.J. et al., "Studies of the HER-2/neu Proto-oncogene in Human Breast and Ovarian Cancer," Science Vol. 244, pp. 707-712, May 12, 1989. Thus the

ability to quantitate the amount of neu HER-2 proto-oncogene and/or its oncogene protein product will allow a clinician to better predict the likelihood of a patient surviving her cancer after completing a selected treatment regimen. By having such information, the clinician will also be better able to select an appropriate treatment regimen to maximize the patient's likelihood of survival.

Brief Summary Text (7):

There would appear to be two ways in which the measurement could be made. The number or amount of neu HER-2 proto-oncogene could be determined in a cell sample using gene probes, which would be expensive and inefficient. Alternatively, the amount of oncogene protein product in the cytoplasm could be measured. While the second choice appears to be more attractive, there are a number of problems encountered with such an approach which prevent easy measurement. The typical tissue specimen biopsied from human breast or ovarian tissue is frozen and then sectioned for microscopic examination. Pathologists favor being able to inspect visually the frozen sections since the portions having malignant cells may be scattered throughout the tissue specimen. It is also difficult to easily determine the locations of cell boundaries in a crowded field because the cancer cells have irregular boundaries. In addition, the monoclonal antibody based stains for visualizing the oncogene protein product work best on frozen sections, as opposed to other types of prepared cell samples. Unfortunately, the sectioned tissue suffers from the problem that while a number of whole cells are present in the section, a number of fractional portions of cells are also present, preventing assaying simply by counting of the cells in an image field of a microscope. It is important to know the sum total of cells being examined because the assay of oncogene protein product is on the basis of the amount of oncogene protein product per cell.

Brief Summary Text (8):

What is needed is a method and apparatus for automatically and quickly assaying the amount of oncogene protein product in the cells of frozen sectioned tissues taken from a human patient.

Brief Summary Text (10):

The present invention provides a rapid and convenient method and an apparatus for practicing the method for determining the amount of oncogene protein product in the cells of a cell sample. The invention is practiced upon samples of tissue taken from sites of suspected malignancies, in particular human breast and ovarian cancers. The tissue sections are cell samples comprising frozen sections of connected cells. Cell samples may also be made from touch preparations, which are made by touching a freshly microtomed or sectioned surface of a piece of frozen tissue to a microscope slide to which the cells cling.

Brief Summary Text (11):

In particular, the apparatus and method employ a mouse alkaline phosphatase based staining system with an anti-rabbit mouse bridging antibody, wherein rabbit antibodies for a protein product of the genes being assayed are connected to the bridging antibody. In particular, the gene may be neu HER-2, the number of copies of which have been found to be an indicator of the long-term survival of a patient suffering from human breast cancer. The alkaline phosphatase antibodies are complexed with an enzyme, in this embodiment alkaline phosphatase. The cells are contacted with the rabbit primary antibody, which binds only to portions of the cytoplasm of the cells having epitopes identifying them as having the protein product of the neu HER-2 oncogene. After applying the bridging antibody, and the alkaline phosphatase antibody, a stain, in this embodiment Naphthol ASTR phosphate and Fast Red KL chromogen, is placed in contact with the cells having the antibody-alkaline phosphatase conjugate bound to their neu HER-2 protein product sites. The alkaline phosphatase catalyzes a chromogen forming reaction only at the areas where it is bound. The catalyzed chromogen forming reaction produces a red chromogen comprised of a red azo dye at the oncogene protein product sites.

Brief Summary Text (12):

The cells also are stained with a conventional stain for DNA, in this instance a thionine stain using the Feulgen technique which yields a blue stain at cellular sites where there is DNA. The image of the cells is magnified in a light microscope and split into a pair of separated images. The separated images are enhanced by a

pair of narrow bandpass optical filters. One of the narrow bandpass optical filters preferentially transmits light having a wavelength at the transmission region of the blue DNA stain thereby producing an optically enhanced oncogene protein product image which only has background and the red chromogen. The background of the oncogene protein product image is composed of the cell nuclei, cytoplasm and the like which have substantially zero optical density. The oncogene protein product sites have a relatively high optical density. Thus the only features which are easily perceivable are the oncogene protein product sites.

Brief Summary Text (13):

The other narrow bandpass optical filter preferentially transmits in the regions of spectral absorption for the blue stain. This filter produces an optically enhanced DNA image of all portions of the cells, with and without neu HER-2 protein product. The apparatus senses the enhanced oncogene protein product image with a first monochrome television camera. The enhanced DNA image is sensed by a second monochrome television camera. Analog signals representative of the images are fed to respective image processors. The image processors convert the analog signals to digitized arrays of pixels which are stored in internal frame buffers. When a tissue section is being examined the apparatus computes a summed optical density of the oncogene protein product image which has high optical density, yielding an area measure weighted by the average pixel optical density for the oncogene protein product in that image field.

Brief Summary Text (14):

In order to avoid the sectioning errors associated with the sectioning techniques used for frozen sections, the invention includes the steps of quantitating a standardized cell sample for DNA in order to determine the linear relationship between the summed optical density of pixels of each cell image in the cell image field having a value indicative of an optical density greater than a selected threshold value. This controls for error which might be introduced by staining variations. A touch preparation is made of cells from the frozen section of the tissue to be examined. This is done by touching the frozen tissue to the warmed slide also having the standardized cells for DNA calibration thereon. The touch preparation comprises a whole cell preparation. In order to obtain the amount of DNA per cell, it is necessary to segregate the pixels associated with each separated cell into separate categories. This is done by the system in conjunction with the human operator. The summed optical density of each of the cell image pixels for each of the sampled cells is also determined in order to determine the average amount of DNA in picograms per cell in the cell sample taken from the patient. This is done in order to remove error introduced by sectioning the tissue sample when the frozen section is made. Thus an average is obtained for the amount of DNA per cell in the cells of the tissue sample. With this information, the clinician then can proceed to the next step in the quantitation of the cytoplasm material, specifically the oncogene protein product. Thus while the whole cell preparation allows an accurate assay of the amount of nuclear material, it cannot be used to assay the cytoplasm. This is because the cytoplasm is relatively fragile and is not completely transferred to the warmed slide in the touch preparation procedure.

Brief Summary Text (15):

Next, a second slide is prepared with a standard cell line thereon having a known amount of DNA per cell and having a known amount of oncogene protein product in the cytoplasm of its cells and the frozen section from the tissue sample taken from the patient. Both samples on the second slide are stained with the thionine stain and the alkaline phosphatase staining system. The first sample is quantitated for both DNA and the oncogene protein product so that the system can create a pair of linear equations relating the optical densities of the pixels sensed by the two optical trains to the known amounts of DNA and oncogene protein product in the calibration sample on the second slide.

Brief Summary Text (16):

The frozen section cell sample containing what may be cancer cells is then examined using the apparatus. Since the cancer cells of the frozen section do not have well defined borders, it is impractical to allow the apparatus and or the human operator to assign areas of the image field uniquely associated with single cells. As a result, the optical densities of the pixels associated with the red chromogen as

detected by the 500 nanometer optical train, and exceeding a second preselected threshold, are summed for the entire image field to provide a summed or total value for the amount of oncogene protein product in the cells in the image field. The total amount of DNA in the image field is also determined by summing the pixels of the image from the 620 nanometer optical train exceeding the first threshold to yield a total for the amount of DNA in the cells in the image field.

Brief Summary Text (17):

The amount of DNA in the image field is divided by the average value for the DNA in the whole cell sample previously determined by examination of the touch preparation thereby yielding the sum total of whole and fractional cells in the image field. The image field cell total for the image field is then stored. The total amount of oncogene protein product is then divided by the image field cell total to yield the amount of oncogene protein product per cell in the image field.

Brief Summary Text (18):

It is principal aspect of the present invention to provide a method and apparatus for quantitating an amount of oncogene protein product for a tissue sample.

Brief Summary Text (19):

It is another aspect of the present invention to provide a method and apparatus for determining an amount of an oncogene protein product in a frozen section of a tissue sample.

Drawing Description Text (2):

FIG. 1 is an isometric view of an apparatus for determining an amount of an oncogene protein product embodying the present invention;

Drawing Description Text (6):

FIG. 5 is a magnified view of the stained cell sample of FIG. 4 as seen through a 620 nanometer narrow band optical filter which yields a DNA or nuclear material image;

Drawing Description Text (7):

FIG. 6 is a magnified view of the stained cell sample of FIG. 4 as seen through a 500 nanometer narrow band optical filter which yields an oncogene protein product image;

Drawing Description Text (14):

FIG. 13 is a depiction of a screen display shown by the system displaying the average number of picograms of oncogene protein product per cell from a frozen section from a patient.

Detailed Description Text (2):

Referring now to the drawings and especially to FIG. 1, an apparatus embodying the present invention and generally identified by numeral 10 is shown therein. The apparatus 10 comprises an optical microscope 12, which may be of any conventional type but in this embodiment is a Reichart Diastar or Microstar. An optical conversion module 14 is mounted on the microscope 12 to enhance optically a magnified image of a cell sample viewed with the microscope 12. The optical conversion module 14, as may best be seen in FIG. 3, has a cell nuclei or DNA sensing means comprising a cell nuclei image optical enhancement unit 16. The cell nuclei image optical enhancement unit 16 has a 620.+- .20 nanometer red narrow bandpass optical transmission filter 18 and a television camera 20 for receiving a filtered image from the filter 18. An oncogene protein product sensing means comprising an oncogene protein product optical enhancement module 22 has a green 500.+- .20 nanometer narrow bandpass optical transmission filter 24 and a television camera 26 and is also part of the optical conversion module 14. Each of the television cameras 20 and 26 generates a standard NTSC compatible signal representative, respectively, of an enhanced DNA or cell nuclear material image and an enhanced oncogene protein product image. An image processing system 28 is connected to the television cameras 20 and 26 to receive the enhanced DNA image signal and the enhanced oncogene protein product image signal and to store a DNA pixel array and an oncogene protein product pixel array therein. The image processor 28 is connected to a computer 32, in the present embodiment, an IBM personal

computer model AT for processing of the DNA and oncogene protein product pixel arrays.

Detailed Description Text (3):

The computer 32 includes system bus 34, connected to the image processor unit 28. An 80286 microprocessor 36 is connected to the system bus 34. A random access memory 38 and a read only memory 40 are also connected to the system bus 34 for storage of information. A disk controller 42 is connected by a local bus 44 to a Winchester disk drive 46 and to a floppy disk drive 48 for secondary information storage. A video conversion board 50, in this embodiment an, EGA board having 256K bytes of memory, is connected to the system bus 34 to control an instruction monitor 52 connected to the EGA board 50. A keyboard processor 54 is connected to the system bus 34 to interpret signals from a keyboard 56 which is connected to the keyboard processor 54. A printer 58 is connected to the system bus 54 for communication therewith. An X Y or image field board 60 is connected to the system bus 34. The X Y board 60 also is connected to a slide holder of the microscope 12 to sense the relative position of a slide 62 with respect to a microscope objective 64 and thus identify a field being viewed. Included are a Y position sensor 66 and an X position sensor 68. The Y position sensor 66 is connected via a communication path 70 to the X Y board 60. The X position sensor 68 is connected via a communication path 72 to the X Y board 60. The microscope 12 also includes an eyepiece 76 in optical alignment with the objective 64 for magnification of light forming an image of a cell sample on the slide 62.

Detailed Description Text (4):

The method of the instant invention is practiced by collecting a cell sample, which may be in the form of a tissue section made from a frozen section or a paraffinized section and having both cell nuclei, cell fragments and whole cells therein. The cells of the cell sample are placed on the slide 62 and fixed thereon. A rabbit monoclonal antibody for a protein product of the neu HER-2 proto-oncogene to be detected in the cells is then placed in contact with them. The monoclonal antibody selectively binds to all points on and within the cells where the neu HER-2 protein product is present. The monoclonal antibody also has bound thereto a bridging anti-rabbit mouse antibody and an alkaline phosphatase complex. The alkaline phosphatase complex comprises an anti-mouse antibody which also specifically binds to the alkaline phosphatase enzyme. The alkaline phosphatase enzyme is bound to the antibody and held through the chain of antibodies to the neu HER-2 protein product in the cells.

Detailed Description Text (5):

In order to view and measure the oncogene protein product sites, a quantity of a mixture containing Naphthol ASTR and Fast Red KL chromogen is applied to the cell sample on the slide. The Naphthol ASTR and the Fast Red KL react to form a red azo chromogen. The usual rate of reaction however is relatively low. The alkaline phosphatase catalyzes the chromogen-forming reaction only at the points where the alkaline phosphatase is localized. Thus, red chromogen is found only at the points in the cells where protein product of the neu HER-2 oncogene is present and the cells are preferentially stained only at the points where they have the oncogene protein product. After a period, any remaining unreacted Naphthol ASTR and Fast Red KL chromogen are removed from the cell sample. The cells are then stained with a thionine stain using the Feulgen technique which leaves a blue stain preferentially bound with the DNA in the cell nuclei. Thus, the DNA is stained blue and the points within the cells having oncogene protein product are stained red.

Detailed Description Text (7):

When the light passes through the filter 18, the filter 18 preferentially blocks light from the blue stained DNA and provides a high contrast cell nuclei image to the camera 20. The optical characteristics of the blue stain and the red chromogen, as well as the optical filters 18 and 24 are shown in the graph of FIG. 7. The camera 20 then generates an NTSC DNA image signal which is fed to the image processor module 28. The image processor module 28 has an image processor 90 and an image processor 92. Each of the image processors 90 and 92 is a model AT428 from the Datacube Corporation. Similarly, the green filter 24 provides a high contrast oncogene protein product image to the camera 26. The camera 26 then feeds the oncogene protein product image signal to the image processor 92. Both of the image

processors 90 and 92 contain analog to digital converters for converting the analog NTSC signals to digitized arrays of pixels which are then stored within internal frame buffers. The internal frame buffers may be accessed via the system bus 34 under the control of the microprocessor 36.

Detailed Description Text (8):

The image of the cell sample viewed through the eyepiece 12 is of the type shown in FIG. 4 having red cytoplasm 99 and a blue cell nucleus 100, red cytoplasm 101 and a blue cell nucleus 102, and red cytoplasm 103 and a blue cell nucleus 104. As may best be seen in FIG. 5, the cells are shown therein as they would appear through the red filter 18, which causes all of the blue stained DNA to darken and appear prominently. As may best be seen in FIG. 6, the oncogene protein product image of the cell nuclei of FIG. 4 is shown therein with the DNA of the cell nuclei 100, 102 and 104 being rendered substantially transparent or invisible by the effect of the 500 nanometer filter 24. The 500 nanometer filter 24 transmits at an optical absorbing region of the red stain and at an optical transmission region of the blue stain. The 620 nanometer filter transmits at an optical absorbing region of the blue stain and at an optical transmission region of the red stain. The cytoplasm 99, 101 and 103 having the red chromogen deposited therein, which is an indicator for the protein product of the oncogene, appears clearly in high contrast.

Detailed Description Text (9):

The image of FIG. 5 is stored in the internal frame buffer of the image processor 90. The image of FIG. 6 is formed and stored in the internal frame buffer of the image processor 92. It may be appreciated that the pixel values for the images may be sliced using standard image processing techniques to increase the contrast between the stained areas and the backgrounds. That is, the areas of high optical density in FIG. 6 the cytoplasm 99, 101 and 103 are shown as being very dense and stored as high optical density pixels, while the background areas 110 may be stored as substantially zero optical density pixels in order to provide a clear threshold or difference between the two areas.

Detailed Description Text (10):

Although the general of processing the images of the stained is disclosed above a more detailed of the invention follows. As may best be seen in FIG. 8, a first slide 148 includes a DNA or nuclear material calibration zone 150 and a whole cell preparation measurement zone 152. In the calibration zone 150 is a cell population having a known quantity of DNA, usually 7.18 picograms per cell nucleus in each of the cells. A whole cell preparation is positioned in the whole cell measurement zone 152 and is prepared by making a touch preparation from a frozen section taken from a human breast cancer tumor. The touch preparation is made simply by touching a warm slide to the frozen tumor tissue and allowing the cells from the frozen tumor tissue to cling to the warm slide. It may be appreciated that all of the DNA or nuclear material, including the entire cell nucleus, from the transferred cells clings to the whole cell preparation zone 152 and is thus pulled intact from the frozen tissue sample, although the associated cytoplasm may be damaged in the transfer. The standard cells in the calibration zone 150 and the cells of the whole cell preparation zone 152 are then stained with a the thionine stain using the Feulgen technique in order to optically enhance the DNA. The system then reads the slide 148 by having it placed on the microscope stage where the image is fed through both of the optical trains 16 and 22. The image received by the camera 20 consists of a darkened area where the DNA has been stained blue by the thionine stain and a substantially clear area outside it. The image is digitized and the resulting pixels are stored. The stored pixels are segregated into separate cell images. The pixel values exceeding the threshold are summed to give summed values of optical density for each of the cells in the calibration zone 150. A similar summing technique is employed for the cells of the whole preparation zone 152. The values are stored and may be displayed in histograms by the system, as shown in FIG. 11. The values also are averaged respectively, for the calibration cells and the whole cell preparation cells. Those averages are used to compute the average value of DNA mass per cell for the cells taken from the biopsied tissue and stained in the whole cell preparation zone 152. The average value of DNA mass per cell is used for later normalization of cytoplasm measurements from frozen sections. Since it is known that the summed optical density from the field from the calibration side is equivalent to a concentration of 7.18 picograms a linear equation can be developed relating the

optical density of the image to the amount of DNA present in the imaged cells. Thus, the optical density of the summed pixels is measured on the right hand side of the slide 150 summed and sum value is inserted into the equation to compute the average quantity of DNA per cell in the frozen tissue section. If the cells are diploid cells, typically the average quantity will be 7.18 picograms. If the cells are tetraploid, which is often common with cancers, the cells will each typically have 14.36 picograms of DNA per cell nucleus.

Detailed Description Text (11):

Once the average amount of DNA per cell nucleus for a number of fields in the calibration slide have thus been determined, a second calibration slide 160 for calibrating the amount of oncogene protein product is then prepared. The second calibration slide 160 includes a calibration portion 162 having a plurality of cells taken from a standard cell line having a known amount of DNA per cell and a known amount of oncogene protein product per cell. An examination zone 164 on the slide 160 has frozen section of the tissue taken from a human patient who is to be evaluated. The standard cells in zone 162 and the sectioned cells in zone 164 are then contacted with an oncogene protein product rabbit antibody which attaches to the protein products of the neu HER-2 oncogenes present in the cells. A bridging mouse anti-rabbit antibody is conjugated with the rabbit antibody. An alkaline phosphatase antibody and alkaline phosphatase are conjugated to the mouse anti-rabbit antibody. Naphthol ASTR and Fast Red KL are then placed on the slide and a red azo chromogen is formed at each of the locations where alkaline phosphatase is present. Thus, the cells in both zones are stained red in the areas in which oncogene protein product is present. The cells also are stained with the thionine stain using the Feulgen technique. This allows the areas having DNA to be identified and measured. The amounts of DNA and oncogene protein product are determined in the same manner as the DNA was quantitated. The system thus has stored therein the average amount of DNA and oncogene protein product per cell for the cells in the calibration zone 162. This allows staining variations to be calibrated out. The distributions of the per cell DNA and oncogene protein product amounts may be output to the user in the form of display information as may best be seen in FIGS. 10, 11 and 12.

Detailed Description Text (12):

Finally, the stained frozen tissue section is examined under the optical microscope. The cell images are fed through the 500 nanometer and 620 nanometer optical trains where respectively, summed optical densities, for the entire image field, of the DNA identified by the thionine stain and the neu HER-2 protein product identified by the red stain are computed. The total number of cells present in the image field under examination is computed by dividing the summed DNA mass by the average amount of DNA per cell, as derived from the measurements made on the whole cell preparation. The summed amount of oncogene protein product is then divided by the number of cells under examination to yield the amount of oncogene protein product per cell, which is output on the display, as may best be seen in FIG. 13. It is this value which will allow the clinician to formulate an appropriate course of action for the patient. Although the invention disclosed herein employs particular materials, it may be appreciated that various other materials may be used. in its practice.

CLAIMS:

1. A method for determining an amount of an oncogene protein product per cell in a cell sample from which a tissue section and whole cell preparation are prepared, comprising the steps of:

measuring an amount of DNA in a DNA calibration sample of cells having a known amount of DNA in each of the cells of the DNA calibration sample;

producing a measured DNA calibration signal in response to said measured amount of DNA;

producing a DNA calibration signal relating the amount of DNA to the measured DNA calibration signal;

measuring an amount of an oncogene protein product in an oncogene protein product

calibration sample of cells having a known amount of said oncogene protein product in the cells of the oncogene protein product calibration sample;

producing a measured oncogene protein product signal in response to said measured amount of said oncogene protein product;

determining the optical density of portions of said whole cell preparation and said tissue section prepared from said cell sample stained with a first stain specific for DNA;

producing a DNA cell sample signal in response to the determination of the optical density of said first stain for said whole cell preparation and said tissue section prepared from said cell sample;

producing a cell count signal for said whole cell preparation in response to said DNA cell sample signal for said whole cell preparation;

determining the optical density of portions of said tissue section prepared from said cell sample stained with a second stain specifically related to said oncogene protein product;

producing an oncogene protein product cell sample signal in response to the determination of the optical density of said second stain;

producing a DNA cell sample amount signal in response to said DNA calibration signal and to said DNA cell sample signal for said whole cell preparation and said tissue section prepared from said cell sample;

producing an average DNA per cell signal in response to said DNA cell sample amount signal for said whole cell preparation and said cell count signal for said whole cell preparation;

producing a total cell number for said tissue section in response to said average DNA per cell signal and said DNA cell sample amount signal for said tissue section;

producing an oncogene protein product copy amount signal in response to said total cell number for said tissue section and in response to said oncogene protein product cell sample signal; and

producing an output signal in response to said oncogene protein product copy amount signal indicative of the amount of said oncogene protein product per cell in said cell sample. PG,24

2. A method of determining an amount of an oncogene protein product per cell in a cell sample, comprising the steps of:

optically enhancing the DNA in a whole cell preparation and tissue section taken from a tissue sample with a first image enhancing material;

optically enhancing the oncogene protein product in the cytoplasm of the tissue section with a second image enhancing material;

determining the average amount of DNA per cell in said whole cell preparation taken from the same tissue sample;

determining the total amount of oncogene protein product in the tissue section;

determining the total amount of DNA in the tissue section;

comparing the total amount of DNA to the average amount of DNA per cell to obtain the total number of cells in the tissue section being examined; and

comparing the amount of oncogene protein product to the number of cells to obtain the amount of oncogene protein product per cell in the tissue section.

3. A method in accordance with claim 2 further comprising the steps of:

calibrating for at least one of the average amount and total amount of DNA by measuring the DNA in a DNA calibration cell sample; and

calibrating for the amount of oncogene protein product by measuring the amount of said oncogene protein product in a oncogene protein product calibration cell sample.

4. An apparatus for determining an amount of an oncogene protein product per cell in a cell sample from which a tissue section and whole cell preparation are prepared, comprising:

means for measuring an amount of DNA in a DNA calibration sample of cells having a known amount of DNA in each of the cells;

means for producing a measured DNA calibration signal in response to said measured amount of DNA;

means for producing a DNA calibration signal relating the amount of DNA to the measured DNA calibration signal;

means for measuring an amount of an oncogene protein product in an oncogene protein product calibration sample of cells having a known amount of said oncogene protein product per cell in the cells of the oncogene protein product calibration sample;

means for producing a measured oncogene protein product signal in response to said measured amount of said oncogene protein product;

means for determining the optical density of portions of said whole cell preparation and said tissue section prepared from said cell sample stained with a first stain specific for DNA;

means for producing a DNA cell sample signal in response to the determination of the optical density of said first stain for said whole cell preparation and said tissue section prepared from said cell sample;

means for producing a cell count signal for said whole cell preparation in response to said DNA cell sample signal for said whole cell preparation;

means for determining the optical density of portions of said tissue section prepared from said cell sample stained with a second stain specifically related to said oncogene protein product;

means for producing an oncogene protein product cell sample signal in response to the determination of the optical density of said second stain;

means for producing a DNA cell sample amount signal in response to said DNA calibration signal and to said DNA cell sample signal for said whole cell preparation and said tissue section prepared from said cell sample;

means for producing an average DNA per cell signal in response to said DNA cell sample amount signal for said whole cell preparation and said cell count signal for said whole cell preparation;

means for producing a total cell number for said tissue section in response to said average DNA per cell signal and said DNA cell sample amount signal for said tissue section;

means for producing an oncogene protein product copy amount signal in response to said total cell number for said tissue section and in response to said oncogene protein product cell sample signal; and

means for producing an output signal in response to said oncogene protein product copy amount signal indicative of the amount of said oncogene protein product per

cell in said cell sample.

5. An apparatus for determining an amount of an oncogene protein product per cell in a cell sample having DNA therein, which DNA is optically enhanced with a first image enhancing material in a whole cell preparation and a tissue section taken from a tissue sample, and having oncogene protein product which is optically enhanced with a second image enhancing material in the cytoplasm of said tissue section, comprising:

means for determining the average amount of DNA per cell in said whole cell preparation taken from the same tissue sample;

means for determining the total amount of oncogene protein product in the tissue section;

means for determining the total amount of DNA in the tissue section;

means for comparing the total amount of DNA to the average amount of DNA per cell to obtain the total number of cells in the tissue section being examined; and

means for comparing the total amount of oncogene protein product to the number of cells to obtain the amount of oncogene protein product per cell in the tissue section.

6. An apparatus in accordance with claim 5 further comprising:

means for calibrating for the amount of both the average amount of DNA per cell in said whole cell sample and the total amount of DNA in the tissue section by measuring the DNA in a DNA calibration cell sample; and

means for calibrating for the oncogene protein product by measuring the amount of said oncogene protein product in an oncogene protein product calibration cell sample.

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TITLE: Dual color camera microscope and methodology for cell staining and analysis

Abstract Text (1):

A method and apparatus is disclosed for use in performing automated classification of cells and other microscopic specimens. The apparatus provides a compact, adjustable assembly that is operable to provide: an operator-apparatus interactive classification system for the cell analysis; alternative techniques for different cells, cytoplasm and cell populations; and enhanced image or color separation and analysis.

Parent Case Text (2):

This is a continuation of application Ser. No. 315,443, filed Feb. 24, 1989 and now U.S. Pat. No. 4,998,284, which is a continuation-in-part of co-pending U.S. patent application Ser. No. 121,674, filed on Nov. 17, 1987, in the names of James W. Bacus and Robert J. Marder, which was entitled "Methods and Apparatus for Immunoploidy Analysis" and now U.S. Pat. No. 5,016,283; which in turn is a continuation-in-part of application, U.S. Ser. No. 927,285, filed Nov. 4, 1986 in the name of James W. Bacus, and entitled "Analysis Method and Apparatus for Biological Specimens" and now U.S. Pat. No. 5,018,209; which is a continuation-in-part of application, U.S. Ser. No. 794,937 filed Nov. 4, 1985, in the name of James W. Bacus and entitled "Method of And An Apparatus for Image Analyses of Biological Specimens" and now U.S. Pat. No. 4,741,043; all of which are commonly assigned with the present application. These previous disclosures are hereby expressly incorporated by reference herein.

Brief Summary Text (3):

In the pathology laboratory, visual observation is the present method for examining cells and tissue. The shape and texture of suspected cancer cells are observed, after staining to contrast and enhance the cells, by a pathologist, primarily through a microscope, who then classifies these cells into a normal category, or one of several abnormal or possibly cancerous categories. Such evaluations are very subjective and do not always differentiate or precisely quantify small changes in DNA, proteins or other substances within individual cells or in very small populations of abnormal cells. For example, these small changes may represent an incipient stage of cancer or a change in cell structure due to treatment of such cancer by chemotherapy or radiation. Therefore, small changes are important in the diagnosis and prognosis of any such diseases.

Brief Summary Text (8):

Therefore, one of the main thrusts of continuing research is the development of stains and staining techniques, which will provide distinguishable optical enhancement or marking of the separate components of cells, for example, the cytoplasm and nuclear DNA, without interfering with the image analysis of the other components. As a practical matter, however, the distinguished components of the cells without the means to analyze these separate characteristics, is of minimal value, although it certainly would be an asset. Therefore, it is necessary to provide the chemical compositions and components to distinguish the characteristics of the various cell components, and to provide the mechanical equipment and electronic networks to analyze the distinguished cell components. Earlier disclosed structures were adequate for transferring and communicating the image to the processing network, however, it is desirable to minimize the physical structure, or at least the apparatus size, for improved manufacture and esthetic appearance, as

well as improving the means for calibration and utilization of the available light energy of the image.

Brief Summary Text (12):

The present invention provides an inexpensive and easily adjustable dual-color sensor system, which aligns and focuses chemically-optically enhanced images of cells and/or materials thereon for viewing by a person; and, for making precise quantitative measurements of the stained materials associated with the cells. Chromogens used in staining are wide band spectra emitters rather than narrow band spectra emitters, and consequently interference between their respective spectrums is a problem to be overcome. Glare is also present, which tends to add light that interferes with precise optical density measurements, and this problem must also be overcome. Good spectral contrast in a stained cell enhances the ability to quantify small changes in DNA proteins or other substances, and this makes it possible to provide the required precision quantitative measurements. Also, signal measurements contain significant electrical noise, which must be removed during electronic processing. This noise deletion is especially required where the signal intensity is not maximized by taking optical density measurements at a measured wavelength. Hence, it is desirable make narrow band wavelength measurements for the respective optical densities at each of two specifically different narrow band wavelengths that do not overlap or interfere with each other.

Brief Summary Text (16):

The present apparatus and techniques further enhance and improve earlier methods and apparatus, as they provide: 1) alternative staining and analytic techniques for different cells, cytoplasm and cell populations; 2) enhanced image or color separation for greater distinguishment by the image processing equipment; 3) a compact, efficient and easily-adjusted image acquisition apparatus to further enhance the image equality and accuracy of the image processing techniques; and, 4) an apparatus for parallel image processing as opposed to the presently available serial image processors. These characteristics thus provide an improved calibration and image processing technique for the analysis of cells or other materials, either biological or inorganic, by image analysis techniques; improve both the method and apparatus for quantitative ploidy analysis of cells through improved image pattern recognition equipment; and provide an enhanced equipment package, which is both visually more appealing and less obtrusive, as well as providing a more technically accurate and efficient utilization of available light energy.

Drawing Description Text (3):

FIG. 1 is a pictorial representation of an image analysis system constructed in accordance with the invention;

Drawing Description Text (5):

FIG. 2 is a functional block diagram of the image analysis system illustrated in FIG. 1, which is adapted to perform the quantitation methods for nuclear DNA in accordance with the invention;

Drawing Description Text (6):

FIG. 3 is a schematic block diagram of the image acquisition apparatus of FIG. 2;

Drawing Description Text (8):

FIGS. 5 and 6 are top perspective and cross-sectional views, respectively, of a microscope slide particularly adapted for use in the image analysis system illustrated in FIG. 1, and having separate areas for calibration cell objects and specimen cell objects;

Drawing Description Text (11):

FIGS. 9, 10, and 11 are pictorial representations of images of a cell population illustrating an unfiltered image, a red filtered image and a blue filtered image, respectively;

Drawing Description Text (13):

FIG. 13 is a pictorial representation of the image monitor display during the selection process and illustrating the marked cells;

Drawing Description Text (17):

FIG. 17 is a system flow chart of the analysis system screen architecture for image analysis system illustrated in FIG. 1;

Drawing Description Text (23):

FIG. 23 is a top plan view of a diagrammatic representation of an alternative embodiment of the image acquisition apparatus of FIG. 3;

Drawing Description Text (24):

FIG. 24 is a top plan view of a diagrammatic representation of the image acquisition apparatus of FIG. 23;

Drawing Description Text (27):

FIG. 27 is an exploded view of the image acquisition apparatus in FIG. 23; and

Detailed Description Text (3):

The apparatus specifically illustrated in FIGS. 3 and 23-28 and the methods described herein can be utilized to develop histograms and other statistical data of cell populations, which may find particular application in the diagnosis and prognosis of diseases. A specific example of this analysis capability is the quantity and distribution of the nuclear DNA, including the distinguishment of particular proteins at protein sites and the DNA nucleus.

Detailed Description Text (5):

In FIG. 1, apparatus 11 includes a digital image analysis and processing system 13, which is broadly shown in FIG. 2. Apparatus 11 includes a high-resolution microscope 15 for viewing magnified specimens on a support or stage which may include a glass slide 14. Microscope 15 has an adjustment or positioning means 70 for focusing optics, such as a condensing lens, on slide 14, and further includes a platform 51 incrementally movable in the X and Y directions through positioning means 12 and 17, respectively, to view the total area of slide 14. Positioning means 12, 17 and 70 may be mechanical adjustment verniers for microscopes.

Detailed Description Text (6):

The specimens, which may be cells mounted on the slide, are in the field of view of the magnifying microscope, and are viewable or reviewable through an imaging system 13 with image acquisition apparatus 18 as well as being visible for analysis in the viewing optics or ocular lens 24. Apparatus 18 is operable to receive the light image at the intensity projected from the specimen field of view. Apparatus 18 thereafter converts the single light beam image into two analog signals [red, blue], which can be individually monitored, sampled and digitally processed by image analysis system 13. Image analysis system 13 is controlled by a system control 22 in the form of a digital processor, such as a personal computer.

Detailed Description Text (7):

An operator, such as a pathologist or laboratory technician, interactively communicates with system control 22 through a keyboard 36. The operator interacts with the system to quantitate nuclear DNA, as well as classify cell objects, by review of two displays or monitors 37 and 62. Image monitor 37, which is the first display, is a conventional RG beam video monitor providing a display through system control 22 and image acquisition apparatus 18, which is the same image provided through a field of view by ocular lens 24. The second display is provided through instruction monitor 62, which is a second conventional RG beam video monitor providing the operator with interactive prompts, images, information and instructions from a system program executed by system control 22.

Detailed Description Text (9):

Image analysis and processing system 13 is illustrated in FIG. 2, which is a functional schematic of apparatus 11. Processing system 13 can analyze a plurality of specimen cell objects or components from the image in the field of view provided through microscope 15 of the plurality of cells on slide or support 14. The image provided through microscope 15 includes light communicated from a variable or fixed intensity source 19 transmitted through slide 14 and thereafter resolved through microscope optic or objective lens 16. As shown in FIG. 1, microscope 15 is a compound microscope with objective lens 16 and ocular lens 24, which may be adjusted

by means known in the art.

Detailed Description Text (10):

Light source 19 transmits light of a relatively broad band spectrum in the visible range or band of light through the cell objects or plurality of cells on slide 14. The optical density of the image converts the light from source 14 into a different intensity output beam communicated to objective lens 16, which differential intensity is dependent upon the percentage of light transmission or, conversely, the percentage of light absorption by the cell object. The visible indication of this phenomenon is presented by the areas of slide 14 wherein no cell objects exist, and therefore these areas will transmit light of high intensity [transparent], whereas areas having nontransmissive or less transmissive objects will appear darker. Generally, an unmodified cell or cell object is relatively transparent and the features of these cells or cell objects are almost indistinguishable. Therefore, the practice of staining cell objects optically enhances the features or objects within the individual cells to highlight or darken them over surrounding features and/or background. In the present invention the stains may be bound to the particular cell by any mechanism, such as absorption, adsorption, ionic bonding, covalent bonding or other method.

Detailed Description Text (11):

The image from each of the cells or cell objects on slide 14 is projected or transmitted to image acquisition apparatus 18 through an optical image splitter 25. Splitter 25 partially reflects the transmitted image to the image acquisition apparatus 18 or the other detector, such as ocular lens 24. Splitter 25 communicates approximately, as a preferred embodiment, 90% transmission of the transmitted light from slide 14 to image acquisition apparatus 18 for subsequent conversion to the optical image for the two scanned electronic signals [e.g., red, blue] through a point-by-point electronic analysis, which represents a monochromatic image of the optical intensity of each of these points in the image communicated to splitter 25, that is, a true color image of the field provided to the analyst at viewing optics 24.

Detailed Description Text (12):

FIG. 3 illustrates an embodiment of the several elements of image acquisition apparatus 18, which includes reflecting mirrors, an image splitter, filters and video cameras, including a digitizing network. As noted above, the light from variable wavelength source 19 is transmitted through slide 14 with a cell or plurality of cells thereon and communicates an image to beam splitter 25, which is mounted in a holder 53. A first true color image is communicated from beam splitter 25 to the viewing optics or ocular lens 24 for focusing and manual review thereof. In addition, a second true color image is transmitted by beam splitter 25 perpendicular to the vertical path from slide 14 to a focusing lens 154 and image acquisition apparatus 18. Lens 154 provides a focused image, that is, a real image, to the image acquisition apparatus 18.

Detailed Description Text (13):

Image acquisition apparatus 18 includes: a plurality of optical elements including a second beam or image splitter 156; reflecting mirrors 158, 160, and 162; and, two monochromatic optical filters 164 and 166. Image acquisition apparatus 18 also includes first video camera 168 and second video camera 170, which receive separate portions of the projected image from second image splitter or beam splitter 156.

Detailed Description Text (14):

The projected image-carrying beam from objective lens 16 and first beam splitter 25 is communicated to both the microscope optics or ocular lenses 24 and to second beam splitter 156. In a preferred embodiment, second beam splitter 156 communicates approximately all of the light above a predetermined wavelength for reflection by mirror 158 through optical filter 164 to first camera 168. Similarly, the light beam wavelengths below the predetermined wavelength are communicated to reflecting mirrors 160 and 162 for reflection through second filter 166 to second camera 170. Filters 164 and 166 are narrow bandpass filters, which filter the light beams transmitted therethrough to substantially provide a light frequency at a specific wavelength. Filters 164 and 166 operate within narrow specified limits and provide an optical block to wavelengths outside this narrow band of wavelengths. Thus, the

light beams provided to first and second cameras 168, 170 are essentially monochromatic images of the field of view on slide 14. Filters 164 and 166 may be changed and selected for either color or wavelength operability, and thus second filter 166, for example, may be a blue filter with a narrow bandwidth different than the width of filter 164. That is, first filter 164 may operate at a wavelength such as 620 .sup.+-. 10 nanometers, and second filter 166 may operate as a blue filter with wavelengths near 500 .sup.+-. 10 nanometers.

Detailed Description Text (15):

Each video camera or sensor 168, 170 is operable to convert the monochromatic, optical, light-carried image of slide 14 on a point-by-point, that is, digital, field into a scanned electronic signal representing the optical intensity or density of each point pixel, in this light image. The output of the first and second cameras 168, 170, which is formatted as a standard NTSC analog video signal, is communicated to an analog or digital converter of a pair of image processing interfaces 21, 23 (cf. FIG. 2), for conversion to a digitized signal, which is received and stored by system control 22. As the image under review on slide 14 is continuously scanned, a real-time image of the area under review is provided by image display 37. In the above-noted Feulgen stain example, dual camera arrangement 168, 170 provides a red color image and blue color image, respectively, simultaneously to the control system, which images may be mixed to provide a combined or focused image of the field of study. Each monochromatic digital image is stored as a 512.times.512 array of pixels, where each pixel has a measured light intensity of 0 to 255 [8 bits].

Detailed Description Text (16):

The parfocal arrangement on either side of first image splitter 25 allows the same or similar image to be reviewed through the ocular lens 24 or on image display 37. The platform 51 may be positioned by the manual X, Y adjustment positioning means 12 and 17 as the operator views a field or reviews a slide to provide a field of interest thereon. Thereafter, the computer-enhanced, digitized image of the selected field is displayed on image display 37 for further analysis. An X-position sensor 26 and Y-position sensor 27, shown in FIG. 2, generate location or position signals to position interface 34 on lines 26a and 27a, respectively, which digitizes these signals to provide apparatus 11 with an accurate coordinate representation of the field of study. Thereafter, the field under review may be reselected at a later date for additional study without scanning the total slide in anticipation of possibly locating the same or similar field of view.

Detailed Description Text (18):

Interface circuits 21, 23, 34, 35, 39, 41, 61 and 71 can be selectively provided on a printed circuit board or boards mounted in the back plane or card connector of a conventional personal computer integral with or forming system control 22. For example, the personal computer can be one manufactured by IBM corporation with a model designation AT, or a similar model compatible therewith. This control system 22 can be operated under a disk operating system such as PC DOS, Version 3.1 or later-issued programming. The system software for the image analysis may be provided on any storage and/or operating means, such as disk drive 75, or a hard disk, and could thus be introduced into the computer operating system by a means such as a floppy disk 77. The system software would be read from disk 77 and loaded into ram 73. Thereafter, the program control would be transferred to system software from the operating system to regulate the various hardware elements of apparatus 11, which were previously set.

Detailed Description Text (19):

Image analysis system 13 provides an interactive program control which projects a number of instruction screens or images on instruction monitor 62 to assist the operator in the quantitation of nuclear DNA found in one or several cells or subpopulations displayed on image monitor 37. Interactive responses by the operator and the menu selections for the different instruction screens will thus function to perform the image analysis of the projected image on monitor 37.

Detailed Description Text (20):

The system functions are more fully illustrated in FIG. 4, where software control logic functions for the hardware of block 80 communicate with software analysis and measuring functions of the system software of blocks 82-96. The software performs an

initialization operation, an interfacing of the operating system function, and overall control of the apparatus by instrument control logic. An image and instruction monitor control logic performs screen handler operations for the instruction screens and digital display of digital images of the specimen for both monitors 37 and 62. Memory and disk storage functions are operated or controlled by the software and memory control logic. Input and output for the interactive responses, as well as report generation, are handled by the keyboard and printer control logic. Data from cameras 168 and 170 as well as position sensors 26 and 27, are handled by image acquisition control logic and position acquisition control logic, respectively.

Detailed Description Text (21):

The control logic of the software forms an operating shell, which is utilized by the analysis and measuring functions of blocks 82-86 to control the hardware of apparatus 11 for performing a particular function. This system provides the following: a patient or cell labeling function 82 to identify the particular tissue samples under study; light calibration and position calibration functions 84 and 86, respectively, which are utilized to provide an accurate reference optical density for a particular field and location of such field with respect to a coordinate origin; control cell calibration 88 operating to provide a datum or reference for the various background stains, DNA index calibration, or other function; a boundary formation 90, allowing the operator to select a reference level for the gray scale value for comparison of either the red or blue image; a selected cell marking function providing marking of the cells identified by cytoplasm optical enhancement in the acquired data function; cell data function 92 for storing of gray scale value of the specimen image measurement; cell classification function 93 operable for the operator to classify the acquired cells, and cell analysis function 94 providing different statistical analyses of categorized data; a utility function 95 providing the needed auxiliary type program for assisting in primary function of image analysis; and, a report generation function 96 to provide hard copy production of the analyzed and compiled data from the system onto or by printer 38.

Detailed Description Text (23):

Apparatus 11 may be utilized in various offices by persons of varying degrees of skill for image analysis. Microscope light source 19 may be adjusted by different operators to vary the light intensity and in addition, the very nature of the lamp may vary from machine to machine as well as depend upon the age of such lamp. Therefore, it is necessary to provide a calibration function for the light intensity to eliminate or minimize errors, or to accommodate the variation in such light intensity. In addition, the staining rate or the quantity of stain utilized may cause a variation in the staining factor, which may be a function of the utilized volume of the particular stain. Such variation in stain levels causes a variation in the gray level output viewed through microscope 15 by cameras 168 and 170, which gray level is utilized to analyze the particular components, such as DNA content. Consequently, apparatus 11 must be calibrated to eliminate the variable differences to provide a true indication of the actual component amount.

Detailed Description Text (35):

In the above-noted Feulgen staining alkaline phosphatase methods, the apparatus for the present method provides a dual filtering method to distinguish the areas stained by the red chromogen [cytoplasm] and the areas stained by the blue Thionin [DNA]. These different images, one provided by the red filter and the other by the blue filter, separate the DNA stained area from the cytoplasm area, which contains the specific antigen, and also separates both areas from other cell or field features. The method uses selective filtering both above and below a wavelength through beam splitter 156, and thereafter filtering these selective wave length images by a color filter technique with a narrow bandpass, which provides maximum utilization of available light intensity and minimizes the light intensity for filtering to each of the separate filter elements 164, 166 to improve their efficiency.

Detailed Description Text (37):

It is noted that Curve A, the Thionin dye curve, has approximately its minimal transmission, or is relatively nontransmissive at approximately a 480 nanometer wavelength, while the fast red dye curve, B, is relatively transmissive at this band range. Later work has indicated that for the present invention the analysis should

be performed at about 500 nm, which conforms with other analytic techniques noted below. Thus, when the image of the cell population is filtered through blue filter 166, substantially all of the area stained with fast red dye will be essentially transparent and substantially all the area stained with Thionin dye will be visible. Therefore, the areas provided with Feulgen stain can be separated from the cytoplasmic stained areas. Similarly, at approximately the band wavelength around band D of red filter 164, the inverse operation is provided. That is, Thionin curve A is relatively transmissive at this bandwidth, while fast red dye curve B, is relatively nontransmissive. Thus, the cytoplasmic areas containing fast red dye are identifiable and are clearly distinguishable from the DNA nuclear areas with the Feulgen stain.

Detailed Description Text (40):

The system software for DNA analysis in the above example can now determine the mass of cellular DNA through optical density measurement of the specimen cells from the Thionin stain through instrument 11. The mass of the DNA of a stained cell object may generally be obtained from its optical density through the Beer-Lambert law which is well known in the art of microspectrophotometry. This analysis provides the mass distribution of a cell or number of cell objects which are available thereafter for analysis by statistical basis, histogram or other analytical format. The spot size, A, noted for the above Beer-Lambert law, is determined by the number of pixels measured by one of cameras 168, 170. The optical density for each pixel is calibrated by adjusting the light level, focus, and reading a reference optical density for the calibration cells 40 on the slide. This calibration allows conversion of the measured light levels for each pixel into an optical density, a dimensionless quantity. The calibration for the extinction coefficient of the above-noted equation is provided by measuring the optical density for a plurality of control cells 40 to provide a peak for the distribution in relative mass units. As the peak DNA content is known for the control cell distribution, the cells in the field of the unknown specimen can be measured using the relative OD units, and thereafter converting these directly into picograms by comparison to the control cell calibration.

Detailed Description Text (41):

FIGS. 9-11 are pictorial representations as follows: 1) FIG. 9 is the true color image of a field of a slide 14; 2) FIG. 10 is an image filtered with the red filter; and 3) FIG. 11 is an image filtered with the blue filter. FIG. 12 is a flow chart of the steps in the method of the present invention to produce quantitation of nuclear DNA.

Detailed Description Text (42):

FIG. 9 illustrates several cells of a subpopulation from one of the fields of microscope slide 14. This subpopulation contains different types, wherein specific cells 202, 204 have been optically enhanced by the alkaline phosphatase staining noted above. All cells 200, 202, 204, 206 and 210 have had the DNA in their nuclei optically enhanced by Feulgen staining with Thionin dye. It is noted that this technique is exemplary and not a limitation. The light beam carrying the image from beam splitter 156 is projected through red filter 164. The image is provided in FIG. 10 and illustrates that only those areas containing fast red dye are visible. These are the cytoplasmic areas 212, 214 of cells 202, 204, respectively, which have been optically enhanced by the staining techniques as they contain a specific antigen that combines with the monoclonal antibody of the alkaline phosphatase stain technique. Cells 202, 204 are different than cells 200, 206, 208 and 210, which are not visible in this image. Further, the nuclei of all cells 200, 202, 204, 206, 208 and 210 are not distinguished or visible in the background, as the optical separation of the Thionin dye and the fast red dye render them essentially transparent.

Detailed Description Text (43):

Conversely, FIG. 11 illustrates the result of projecting the image from beam splitter 156 through blue filter 166, wherein all the nuclei of the several cells, e.g., 216, 226, from the cell population are visible. In this image, the blue filter provides an exclusion of the stained cytoplasmic areas, which are not nuclear stained, and are thus optically different, although stained, and thus transparent.

Detailed Description Text (44):

The areas stained above the threshold set for each filtered image can then be combined by digital overlayment of the DNA image upon the cytoplasmic image, which presents a clear image of the DNA nuclear areas to monitor for typing and analysis where certain cells 302, 304 are marked as to type by an identifying cytoplasmic ring or crescent on the nucleus in FIG. 13. The DNA analysis then proceeds by interactive classification of each cell in the image displayed in the image monitor 37. Specifically marked cells 302, 304 can be included in any class, excluded from any class, or separately classified. Further, it is evident that different optical enhancements and filterings will give rise to different cell typing and increased sensitivity of the classification process.

Detailed Description Text (45):

The method of measuring and analysis of DNA using the marking technique of the invention is more fully illustrated in FIG. 12. In a first step in block 250, a slide containing control cell objects and specimen cell objects is stained with the above-noted alkaline phosphatase technique utilizing fast red dye. The monoclonal antibody is specific against the cytoplasmic antigen, for example, leukocyte common antigens or Cytokeratins. The next step in the process is to stain the slide 14 with the above-noted Feulgen process, utilizing Thionin dye as noted in labeled block 252. After mounting, slide 14 is placed on platform 51 of instrument 11 and positioned to provide a clear field on image monitor 37. The light level is then set, as provided in step or block 254 of the flow chart.

Detailed Description Text (46):

Platform 51 is adjusted or traversed to control cell 40, or an image of a subpopulation of the control cell or cells appears on monitor 37 [block 256 of FIG. 12]. The image is that of the filtered image [red] showing only Feulgen stained areas. The amount of staining to determine the DNA index therein, for determination of the mass through optical density, is found by measuring the optical density of the control cells [block 260]. Generally, the calibration is repeated to obtain an accurate measurement and assessment of the calibration and the process is repeated merely by iterating through the steps in blocks 260 and 262. In block 262, the platform 51 may be manually adjusted to another location to provide a second field of control cells.

Detailed Description Text (48):

A cytoplasmic image of the specimen field may be obtained utilizing the blue filter [block 266] and its boundary [block 268]. Similarly, a DNA image of the specimen field is provided through the red filter [block 270] and its boundary set [block 272]. These filtered images are real-time images of the field and may be constantly updated through image acquisition means 18 of system 11. Apparatus 11 combines the two filtered images [block 274] to mark the selected cells on image monitor 37 while displaying the nuclear DNA area. The analysis program then proceeds to the classification step [block 276]. In the classification mode, the image acquisition and combination [marking] ceases and a static or fixed image is projected on image monitor 37.

Detailed Description Text (49):

The cells in the image on monitor 37 are classified by type through an interactive process with an operator. Each cell is noted by the apparatus and the operator selects a classification for the separate cell using nuclear morphology and cytoplasmic markings of the combined image. Classified cells are then measured for the cell component, such as DNA content, [block 278] and the results of the measurements may be displayed [block 280]. This measurement display can be accommodated in several forms and with statistical analyses of the different classifications or combinations of such classifications.

Detailed Description Text (50):

The measurement step can include more than the cells in a single field simply by iterating through the steps noted in blocks 280-284 in FIG. 12. The operator may manually move platform 51 to another specimen field, and the marking and imaging steps may again be repeated as described above. The accumulated data in the measuring steps for the new cell populations is compiled with that of the previously developed cell population data. The display step noted in the above description can

be delayed until a significant or required amount of data is accumulated, or display of each iteration may be provided at the option of the operator. In addition, the operator may elect to bypass setting the cytoplasm boundary and DNA boundary after they have been first set for a specimen image.

Detailed Description Text (53):

The calibration menu, FIG. 19, provides means for setting the current image or field location as the origin, by zeroing a pair of location registers in the software.

Detailed Description Text (54):

The measure function A42, controls the control cell or control object calibration to normalize the staining factor. During control cell calibration, the operator moves the microscope stage by adjustment of the X and Y knobs 12, 17, respectively, to shift cell objects 40 into the field of view on image monitor 37.

Detailed Description Text (55):

Additional functions are provided on the various screens for various operations of the process. These include: the X-Y function to aid in the positioning of platform 51; the FOCUS-1 function A40 to provide color enhancement to the image; the analyze function, which provides a menu function shown in FIG. 22, to perform the DNA measurements on the cellular material; the check light-2 function A82 calculates the light level of the current image; the select-second function A84 permits the user to select the second peak on the histogram displayed on an analysis screen; the classify function A78 allows the user to classify the cells or objects on image monitor 37; the display X, Y function A88 changes the display from the analysis screen to the X, Y field coordinates screen; the clear-2 function A90 clears all analysis-related areas of data; the focus-2 function A80 provides color enhancement; the area 1-2 function A86 allows the user to specify two areas in the histogram displayed on the analysis screen.

Detailed Description Text (58):

In FIG. 3, the image acquisition apparatus 18 encompasses a beam splitter 25 secured and mounted in a holder 53, which holder also secures focusing lens 154. The focused and magnified image provided from the light source 19 and microscope 15 enters beam splitter 25 mounted in holder 53. Beam splitter 25 provides an image for manual observation of the cell under analysis through the viewing optics or ocular lens 24 as well as apparatus 18. In FIG. 3, holder 53 and beam splitter 25 are generally centrally located to project a magnified image light beam along the longitudinal axis of the image acquisition apparatus 18 and has second beam splitter 156 likewise aligned on this longitudinal axis. This FIG. 3 system provides a light path to the cameras which is longer than desirable, as it requires reflecting a light beam of lower intensity than the beam from first beam splitter 25. Consequently, an alternative path is provided, as noted in FIG. 23, to initially reflect the higher intensity polychromatic beam, thereby providing a more uniform beam intensity to both video cameras. That is, second beam splitter 156, which may be a dichroic beam splitter, divides the higher intensity first light beam into two light beams of approximately equivalent intensities. This arrangement provides more closely matched beams at the video cameras, which reduces the adjustments required to balance the overall image system. A further benefit of this structure is a more compact assembly with easily adjustable components in the optical circuit.

Detailed Description Text (59):

In FIG. 23, image acquisition apparatus 18 is illustrated in a generally rectangular outlined manner with a longitudinal axis 19, which is parallel or generally parallel with the longitudinal axis 169 of first video camera 168 and axis 171 of second Video camera 170. In this alternative embodiment, focus lens 154 is positioned in holder 53 at a location perpendicular to longitudinal axis 19 to receive light beam 153 carrying the magnified image from beam splitter 25. This image or light beam 153 is projected through focus lens 154 to first mirror 160, for reflection to second mirror 162 and second beam splitter 156. In this configuration, the image or light beam 153 is projected normal to axis 19, reflected at first mirror 160 at about a right angle to second mirror 162 and again reflected at a right angle for communication of the magnified image to the second beam splitter 156, which second reflection from second mirror 162 is illustrated along a line parallel to the image line of first light beam 153. Thereafter, second beam splitter 156 splits the first

light beam 153, which has only suffered attenuation from reflection, into a second light beam 157 and a third light beam 159.

Detailed Description Text (61):

As an example but not a limitation, light beam 157 is below a predetermined wavelength and is transmitted through first monochromatic optical filter 164 for communication to and reflection from third reflecting mirror 158, which reflects light beam 157 at about a right angle, that is generally parallel to longitudinal axis 19, to first video camera or sensor 168 at the first camera front plane 173. Third light beam 159 is reflected by splitter 156 through second monochromatic light optical filter 166 to second video or sensor camera 170 at its front plane 175. In this configuration it is noted that the light beams are projected or reflected at right angles or generally right angles to the intersecting or reflecting planes such that the beam essentially travels either parallel to or perpendicular to longitudinal axis 19 of the image acquisition apparatus 18. First monochromatic optical filter 164 and second monochromatic optical filter 166 are in proximity to the second image beam splitter 156, which arrangement provides the maximum intensity of the split image light beams and minimizes the potential attenuation suffered by communication of such beams by reflecting or filtering devices.

Detailed Description Text (62):

As noted above, second image beam splitter 156 splits first light beam 153 into a continuous spectrum with a bandwidth below a predetermined wavelength, which is second light beam 157, and another continuous bandwidth spectrum above the predetermined wavelength, which is third light beam 159. These light beams are projected or communicated to first video camera 168 or second video camera 170, respectively. First light beam 157 is provided to first camera 168 at its front plane 173 through first monochromatic optical filter 164 and mirror 158. First filter 164 is specifically chosen to provide an approximately monochromatic optical image at a predetermined wavelength, that is, it filters out wavelengths outside the first predetermined wavelength. Similarly, the second monochromatic optic filter 166 filters or selects a section of a light beam bandwidth at a predetermined wavelength for communication to second video camera 170 at its front plane 175. As the bandwidths of the light beams have been narrowed by second splitter 156, the filters are more efficient in selectively providing the selected wavelength light to cameras 168, 170.

Detailed Description Text (63):

In FIG. 23, the separation distance from second image beam splitter 156 to the third reflecting mirror 158 is noted as X; the distance from the third mirror X to front plane 173 of first video camera 168 is Y; and, the separation from second image beam splitter 156 to the front plane 175 of second video camera 170 is denoted as Z. In this configuration, the sum of the beam path or distances $X + Y$ is equal to the distance Z.

Detailed Description Text (64):

The image from the second beam splitter 156 to second camera 170 in this embodiment, is only projected through the second optical filter 166 and thus, the image or rather the intensity losses to the second video camera 170 due to reflection or interference are minimized. In addition, optical filter 164 is in closer proximity to second image beam splitter 156 to enhance the image to first camera 168 by filtering a light beam which is not attenuated by distance or intermediate reflection or diffraction.

Detailed Description Text (65):

In this embodiment, which is illustrated in detail in FIGS. 24-27, image acquisition apparatus 18 includes housing 402 which has a bottom panel 404, top panel 406, front panel 412, rear panel 413 and, first and second sidewalls 408, 409. In FIG. 27, which is an exploded view of apparatus 18, housing members 404, 406, 408, 409, 412, and 413 cooperate to define an enclosure or chamber 410 wherein the components of image acquisition apparatus 18 are mounted and operable. Bottom panel 404 is essentially utilized as the mounting base for securing or maintaining the various operating components of image acquisition apparatus 18. In this illustration, bottom panel 404 includes front panel 412 with a base 414 to receive the holder 53 for first beam splitter 25 and focusing lens 154. Mounting base 414 defines a plurality

of threaded holes 416 in alignment with through bores 418 of holder 53 to receive securing means 420, shown as threaded screws, which mate with threaded holes 416 to secure holder 53 on bottom panel 404.

Detailed Description Text (66):

Bottom panel 404 includes first pivot socket 422 and second pivot socket 424 as well as first threaded securing port 426 and second threaded port 428 (cf. FIG. 25) communicating with first and second pivot sockets 422 and 424, respectively. First reflecting mirror 160 is mounted on first mounting base 432, which has a first pintle 436. Second mirror 162 is similarly mounted on second mounting base 434 with second pintle 438 affixed thereto. First pintle 436 and second pintle 438 are positioned in first and second pivot sockets 422, 424, respectively, and first and second mirrors 160, 162 are rotatably adjustable in the sockets for positioning the reflection of the magnified image received from focused lens 154. After alignment of mirrors 160, 162, securing means 430 in ports 426 and 428 contact first and second pintles 436 and 438 to secure the mounting bases and mirrors.

Detailed Description Text (74):

First video camera 168 is mounted in and longitudinally movable in channel 554. Camera 168 is slidable along axis 109 in channel 554 to focus the image projected on screen 37, and it may be secured in channel 554 by a pressure plate 594 interposed between the camera and fifth and sixth upright brackets 548 and 550 to secure camera 168 against middle or central brackets 540 and 542 respectively. Pressure plate 594 is secured against the camera 168 by a pair of threaded locking means or bolts 600 extending through passages 596, 598 in fifth and sixth upright brackets 548, 554, respectively. In addition, camera 168 may adjust its signal level with a pedestal adjustment means 602 and an electronic gain adjustment means 604.

Detailed Description Text (75):

After assembly of the above-noted elements of acquisition apparatus 18 on bottom panel 404, first and second cameras 168, 170 are positioned in housing 402 and a magnified image is transmitted to the cameras in their assembled positions by adjustment of mirrors 160, 162 and 158 as well as by adjustment of second beam splitter 156 and the optical filters 164, 166. First and second reflecting mirrors 160, 162 are rotatably adjustable on pintles 436 and 438 in sockets 422 and 424, respectively, to communicate the first light beam 153 from first beam splitter 25 to second beam splitter 156. Second beam splitter 156 is adjustable in recess 440 in a horizontal plane generally defined by bottom plate 404. Gimballed plate 450 may be adjusted by pivotal motion about pivot means 462 against the force of lateral bias means 486. It is recognized that the several securing means utilized to secure the gimbal plate in its desired position are not engaged during the adjustment. The vertical adjustment of second beam splitter 156 is provided by loosening or tightening the securing means 480, 482 and 484 to allow the beam splitter 156 to slightly rock or rotate on spherical bearing 464 against the bias force of vertical biasing means 466, 468 and 470. Finally, third reflecting mirror 158 is adjustable by adjustment means 501 and 503, which provide vertical and horizontal adjustment to generally center the light beam 157 on lens plane 173 of first camera 168. It is further recognized that mounting base 494 may be slightly adjustable initially as the third mirror gimbal assembly is affixed to bottom plate 404. In FIG. 23, the monochromatic optical filters 164 and 166 are placed as closely in proximity to second beam splitter 156 in order to minimize the intensity losses of the light beam signals projected thereto from beam splitter 156. In addition, light beam paths 157, 159 from second beam splitter 156 to first camera 168 and second camera 170, respectively, are approximately equivalent distances, to provide equivalent light intensities to both cameras, which assists in the focusing of the cameras.

Detailed Description Text (77):

The adjustment or focusing procedure for cameras 168, 170 is similar to the procedure for the cameras of earlier models of analysis equipment. A calibration image is provided from microscope 15 through first beam splitter 25 and focusing lens 154 for reflection by first mirror 160 and second mirror 162 to second beam splitter 156. The split beam or beams 157, 159 from splitter 156 are communicated to first video camera 168 and second video camera 170 through first and second optical filters 164, 166, respectively. The first and second image outputs from video cameras 168 and 170 to the digitizing means is projected on screen 37 for review and

calibration. Thus, the image in first camera 168 can be individually focused by slidable camera movement along axis 169. Second camera 170 and the image projected therefrom to screen 37 may be focused and adjusted by rotation about or slidable motion along axis 171, which camera 170 is thereafter secured in position. After the camera adjustments, the clamping and support means secure cameras 168, 170. Exemplary of adjustment means are front support 556 and rear or back support 576 wherein securing means 582 may be loosened to allow camera 170 to be rotated about its axis 171.

Detailed Description Text (78):

Apparatus 11 is generally utilized for analyzing biological cell specimens. The image from objective lens 16 of microscope 15, which is the projected and magnified image of the calibration or cell slide, is projected upward to beam splitter 25 to provide the image to both the ocular lens 24 and focal lens 154, which may have a fixed or variable focal length. Magnified image light beam 153 after focusing through lens 154, which is a real and focused image in contrast to an unfocused and virtual image, is reflected by first and second mirrors 160, 162 to the second beam splitting means 156. First beam 153 is divided or split by the second beam splitter 156 into a second light beam 157 and a third light beam 159 with about equal intensity for projection and communication to and through first monochromatic optical filter 164 and second monochromatic optical filter 166.

Detailed Description Text (80):

It is known that cells may be single cell structures but most have at least two components, such as a nuclear DNA with a DNA nucleus and a protein at a protein site associated therewith. Analysis and review of these cells and their various components thus requires selective staining techniques for analysis, for example pathology studies. In some instances, the staining techniques, as noted earlier in the application, provide staining or distinguishing characterization of certain components of the cells, that is, a first cellular component such as a DNA nucleus and a second cellular component such as a protein at a protein site. These are merely exemplary of components of the cells that are available for staining and identification. Among the known proteins are receptors, enzymes, structural proteins, glycoproteins, and lypoproteins. Other potential cell components include nucleic acids (e.g., RNA, mRNA, rRNA, and DNA), hormones (e.g., steroids, estrogen, peptide hormone, and progesterone), and lipids, which are noted as components of the cell membranes. It is appreciated that these are merely representative of cellular components which may be available for staining and identification by an existing or later discovered staining technique. However, in order to observe and identify these various components, and thereafter utilize the gathered information, it is necessary to first present them in an identifiable fashion. Therefore, specific staining techniques are utilized to provide enhanced optical contrasts for combinations of the various components, each component being responsive to a particular stain or staining technique. The following listing provides examples of stain combinations, which are operable to provide contrasting overlapped curves, as in FIG. 8, with graphical plots of transmittance as a function of wavelength where the maximum for at least one plot is adequately separated from the second curve for analysis.

Detailed Description Text (81):

The above-noted stains and staining techniques provide enhanced contrast between the various components to be identified and, in addition, have optical characteristics which may be matched or corrected with monochromatic optical filters to provide a discernible and identifiable cell component. In a preferred embodiment, the selected stains react with two separate cellular components to provide a contrasting image at two distinct limited ranges of wavelength or spectral bandwidths. This provides the image acquisition apparatus with the opportunity to segregate each preferred wavelength and to provide the beam splitter and monochromal optical filters the least interference or most narrow bandwidth image signals for resolution of the signals to the selected or desired wavelengths for analysis by one of the video cameras or sensors.

Detailed Description Text (82):

Cells, which have been stained to highlight or characterize an individual cell component, are not as formidable an analytical task as an unstained cell, as the stained component will generally be visibly apparent through a microscope. The

optical equipment or optics related to such analysis or recognition are well known. It is recognized that a stained cell exhibits specific optical characteristics, such as transmittance, and more particularly it may have a maximum transmittance at a first wavelength (implying relative transparency) and a minimal transmittance at a second distinct wavelength. In the case where only a single stain is utilized, either of the stained cell components could be analyzed at any wavelength along their curve with some reasonable effort. However, when two stains are simultaneously utilized on a cell to stain or combine with particular cell components, their overlapping spectral emissions may interfere with each other. As a consequence, extensive filtering, both optical and electronic, may be required to discern the specific parameter, characteristic or cell component under investigation. Therefore, it is desirable to provide stains which exhibit contradictory or converse transmission characteristics at approximately the same wavelengths, which in an ideal condition would imply minimum transmittance of a first stain at the maximum transmittance of the second stain, as implied in FIG. 8 at about 500 nanometers. Communication of a narrow band visible light beam through a monochromatic optical filter generally provides a light beam at about a fixed wavelength, which can be selected to correspond to the maximum-minimum point noted above. The image contrast from these two competing stains would provide a more easily analyzed cell component whose stain characteristic is at its minimum transmittance. Similarly, communicating the light beam through a second optical filter (about 650 nanometers in the Example of FIG. 9) would provide a different spread between the spectral transmittance curves.

Detailed Description Text (83):

In the analysis of cells there are different imaging methods, that is different reasons for cell analysis including quantitative immunoploidy (QIP) studies, quantitative nuclear antigen (QNA) analysis and proliferation index. As an example, in an immunoploidy analysis, a type two stain (Feulgen DNA stain) is applied to the nucleus (cf. FIG. 8), and a type one stain of the red-chromagen-alkaline phosphatase variety is applied to outline and distinguish the cytoplasm. At 620 nm in FIG. 8, after acquiring and recombining the separate images, the nucleus of the cytoplasm has approximately 100% transmission and essentially a mask is provided and the nuclear DNA is enhanced for review. In some cases the review is conducted by optical density measurements, which were discussed above, for analysis of the DNA mass. Alternatively, the separation of the curves at approximately 500 nm allows a study of the cytoplasm protein sites, which are distinguished by the greater absorbency of the illustrated red chromagen stain.

Detailed Description Text (84):

In the quantitative nuclear antigen measurements, the cell component interest may be restricted to the nucleus and more specifically nucleus components. The components may be nonspecific nuclear proteins and specific proteins. As illustrated in FIG. 29, the nonspecific proteins or all nuclei are stained with a type one (acid-base reaction) stain, such as ethyl green, and the specific proteins or antigens are stained with a type three stain, such as diaminobenzidine (DAB). The immunohistochemical stain (type three) has an antibody stain that is specific to an antigen of some of the nuclei, such as an estrogen receptor, progesterone receptor or a proliferating cell antigen. After application of the peroxidase which colors the antigen, light transmitted through the stained cell will be absorbed or transmitted at different rates as noted in FIG. 29 at the 500 nm and 620 nm ranges. As noted in this Figure, at 500 nm the ethyl green stained nucleus has essentially 100% transmittance, and the DAB stained nuclear component absorbs a large percentage of the light. Therefore, the DAB stained component (red channel) shows through. This method provides a mask for the nuclei and the dark objects are distinguishable. At the second or blue channel, the actual intensity or stain density of the nuclear components is measured, that is, the area of the mask that is immunohistochemically stained. The red channel is thus distinguished, as there is no output from the masked component with 100% transmittance. Earlier analytical color camera developments did not utilize two different stains.

Detailed Description Text (86):

The above-noted examples clearly indicate and exemplify methods of cell analysis and more specifically, quantitative analysis of cell components with true optical and mass determination. The first monochromatic image above provides a means to provide

the quantitative analysis of at least the first component, and the second monochromatic image is invaluable in the identification of other cell components.

Detailed Description Text (87):

The present invention provides both method and apparatus to correlate various stain pairs with the required monochromal optical filters. The two-color camera system is principally used in immunohistochemistry stain applications. These are very often two-color stains, that is the colored antigen/antibody/stain complex vs. cells in tissue compartments without antigen and stained with a counterstain. As it is frequently desired to quantitate either the material counterstained or the antigen/antibody complex, via measurement of cell stain content, it is helpful if at least one of the staining components does not have a spectral overlap at the sensing wavelength of one of the image sensors. It is preferred to make measurements at approximately the peak transmission value, preferably 100% transmission, as that provides the biggest contrast to the surrounding light spectra and cell components in the cell matrix. Further, the visible light spectrum is not a very broad band region of the total light spectrum, and analysis of cells using a second stain may introduce interference of the overlapping spectra, as these stains tend to be broad band spectra or have broad band spectral outputs. In addition, wide bandpass spectral filters of standard color cameras or sensors contribute to any glare problem during the analysis. Thus, the narrow bandpass filtering reduces an inherent problem of earlier structures. In addition, use of a standard color camera for review of multi-stained cells invites an inherent problem in the output and review of the cells, that is, pixel-to-pixel alignment of the separate images cannot be accommodated as the same regions are not projected in each pixel in the three (usually red, green, blue) colored images provided from a color camera. As a consequence, the images under review are not accurately or consistently reviewed during the analysis, whereas the inventive structure provides for pixel-by-pixel calibration, alignment and cell review.

Detailed Description Text (88):

The image acquisition apparatus noted above provides a means to split or divide the amount of light communicated to each of the individual cameras through the use of a beam splitter, which is preferably a dichroic beam splitter. This attenuation of the light or light transmission may be accomplished at a specific wavelength, as the beam splitter is transparent to a band of wavelengths either above or below the selected wavelength thus allowing that band of light to be transmitted through the splitter; and, it reflects the light beam at the other wavelengths above or below the preselected wavelength and thus limits the band width of the light transmitted to each of the monochromal optical filters. Whether the light beam communicates to the beam splitter is merely split or split at a specific wavelength, the optical filters selectively limit the light transmitted to the cameras to a specific or narrow range of wavelengths for analysis. Provision of a narrow bandwidth light beam to the optical filters improves their efficiency by limiting the background "noise" to be screened from the desired light signal.

Detailed Description Text (89):

Second beam splitter 156 may be exemplary of a dichroic beam splitter, and, for example, may provide transmission of all wavelengths of light above 550 nanometers and reflect all wavelengths below 550 nanometers. Therefore, marking a cell, such as those selected from a patient for cancer analysis, by utilizing one of the above-noted stain combinations will cause the first stain to mark the nucleus and the second stain to mark the associated protein at a protein site. Individual light beams at different specific wavelengths for the stained cell are consequently provided to the first and second camera. Thus, each of the individual components after appropriate optical filtering are analyzed at one of the first and second channels, which is dependent on the light at a wavelength either transmitted or reflected thereto. The electronic light signal from the camera is transmitted to the computer analysis system for conversion to a digital signal, storage and analysis of each of the first and second channel signals and/or combination of these two individual signals or figures for comparative analysis in an overlapping array.

Detailed Description Text (90):

The two-color camera analytical system of the present invention is matched to the staining spectra of readily available, common acid-base and immunohistochemical

stains. The spectral wavelengths chosen match at least one region of 100% transmission of one paired stain component. At the same time, the narrow bandpass filters reduce glare. This is important for purposes of accurate densitometry measurements of the stained substance. By comparison the sensing spectra of typical solid state ordinary "color" cameras use three broad band spectra (these are chosen to match the color visual receptors of the human eye). These overlap with the broad-band, two component, cell staining spectra, e.g., FIGS. 8, 29, and 30; and the wide bandpass spectral filters of the standard color cameras contribute to glare. In addition, the color filtering of standard color cameras occurs on separate pixels (different lines of video) on the solid state sensing chips. Thus, the pixels are not from the same regions in the three-colored images of a color camera.

Detailed Description Text (96):

Beams 157 and 159 should be noticeable on the noted target or mask. First mirror 160 should be rotated such that the beam to camera 170 is a full circle and not "clipped," on either the left or right side. If the beam is clipped on the top or bottom, first beam splitter 25 must be adjusted by rotating screws 420 until the beam is centered. Thereafter, first mirror 160 should be locked or secured in place. The optical equipment should not be contacted by fingers during the adjustment period. Similarly, second mirror 162 is rotated to provide an unclipped image to the mask, and first beam splitter 25 is adjusted to properly center the third beam. Subsequently, second mirror 162 is likewise secured by locking screws.

Detailed Description Text (97):

The target can now be removed, and the grid image will be projected on face plate 175 for centering. The elevation or vertical alignment of the projected image on face plate 175 of second camera 170 may be centered by rotation or adjustment of locking screws 480, 482 and 484 of second beam splitter 156. Adjustment of the grid image on face plate 175 across its horizontal plane, that is, left to right, is accommodated by rotation of second beam splitter 156. Adjusting screw 192 securing locking washer 488 is loosened, and turning adjustment screw 493, which contacts the beam splitter mounting pedestal 450, moves pedestal 450 in recess 440. After proper alignment, securing screw 492 may again be tightened to secure washer 488. The grid image, which is generally red, on face plate 173 of first camera 168 may be adjusted by adjustment of screws 501 and 503 on the reverse side of third mirror 158. Bottom screw 503 will move the image left and right across the face plate 173, whereas top screw 501 will adjust the image vertically up or down and will also rotate the image slightly. Thus, screws 501, 503 are utilized to center the image on face plate 173. It is noted that rotation of the image will require repetition of the earlier adjustment of the first beam splitter 25 and also correct centering of the grid image on face plate 175 of second camera 170.

Detailed Description Text (98):

If finer adjustment of the coupler is required, an adjustment cover is placed on the apparatus 18, and, if removed, the eyepiece is returned to the coupler. The grid slide is focused and centered in the eyepiece. Access to front and back rotation lock screws 604 and 582 of second camera 170 is provided through the alignment cover. These screws are loosened and the image from second camera 170 is displayed on monitor 37. Thereafter, second camera 170 is slidably moved along its axis 171 until the monitor image is properly focused. Subsequently, locking screws 600 for first camera 168 are loosened, and the image from first camera 168 is displayed on monitor 37. First camera 168 is similarly slidably moved along its longitudinal axis 169 to focus the image on monitor 37. Locking screws 600 are thereafter tightened, and cameras 168, 170 are now in parfocal. The image on monitor 37 from first camera 168 is centered by adjustment of screws 501 and 503 on third mirror 158. In this adjustment, there should be an equal number of lines on either side [and top to bottom] of the center heavy lines of the grid pattern. The image from second camera 170 is again displayed on monitor 37 and this image is centered by rotation of adjusting screws 480, 482 and 484 of second beam splitter 156 for left-to-right adjustment. Vertical adjustment of the image from camera 170 is again provided by loosening screw 492 and rotating adjusting screw 493 to adjust pedestal 450 in recess 440 prior to resecuring screw 492. The projected grid image is again checked for proper alignment in both vertical and horizontal directions. The images from both first camera 168 and second camera 170 are now projected on monitor 37, and the images are aligned such that there is no greater than 1/4 line width of grid pattern

between the superimposed images. Vertical and horizontal adjustments are provided by adjusting screws 501 and 503 of third mirror 158, whereas rotational adjustments are made by physically rotating second camera 170. Second camera 170 is rotated until the grid lines of both images are parallel, however, care must be utilized to avoid changing the focus of second camera 170. Both front and back rotation lock screws 604, 582, respectively, are thereafter secured to maintain second camera 170 from further rotation. Third mirror 158 is now adjusted to provide vertical and horizontal alignment of the grid on the monitor. The rotational and vertical-horizontal adjustment may require repetition to achieve proper alignment. The grid slide on the microscope is now moved to a clear area, and the microscope diaphragm is adjusted for proper focus, size and centering. A proper light intensity is provided by attaining a reading of approximately 200, which is a comparative gray-scale reading on the second camera 170, and the gain adjustment 604 of first camera 168 is adjusted to similarly give a light reading of 200. The apparatus cover 402 is reinstalled and the apparatus is prepared and ready for operation.

Detailed Description Text (99):

It is further considered that the above apparatus is equally applicable to a fluorescent antibody stained cell. As an example, when infectious agents such as viruses and bacteria, and other antigenic materials which are principally of a protein nature, invade body tissue, soluble substances are produced which specifically react with these alien materials. The soluble substances are called "antibodies" and materials which elicit their production are called "antigens." Antibodies can be coupled to fluorescent dyes such as Fluorescein. Antibodies labeled by fluorescent dyes are called "fluorescent antibodies" and are utilized as immunospecific stains for the detection of antigens in cells and tissues. The marked regions in a cell are seen as a characteristic color when the cell section is examined with a fluorescent microscope. This method of fluorescent microscopy may also be operable with the above-noted apparatus. Transmission microscopy was utilized for the examples in the particular description noted above. However, it is appreciated that the present apparatus may be applicable to the fluorescent microscopic analysis at specific wavelengths. A proper staining agent must be utilized to mark either the nucleus and/or protein at a selected protein site when either the fluorescent or transmission microscopy techniques are utilized with the apparatus and system of the present invention.

Other Reference Publication (13):

Oud et al., "DNA and Nuclear Protein Measurement in Columnar Epithelial Cells of Human Endometrium," Cytometry, vol. 7, pp. 325-330 (1986).

Other Reference Publication (15):

Gunzer et al., "A Note on the Usefulness of Multi-Color Scanning and Image Processing in Cell Biology," The Microscope, vol. 24, pp. 39-44 (1976).

Other Reference Publication (16):

Oud et al., "The use of Light Green and Orange III . . . of protein and DNA," Histochemistry, vol. 80, pp. 49-57 (1984).

Other Reference Publication (21):

Sklansky et al., "Biomedical Image Analysis", Chapter 26, Handbook of Pattern Recognition and Image Processing, 1986, pp. 629-647.

CLAIMS:

1. A method of analyzing cells, having at least a first cellular component and a second cellular component, said method comprising:

chemically-optically enhancing the cells with at least a first and a second spectral stain material;

each of said spectral stain materials including the first and second spectral stain materials combining with one of said cellular components to form at least a first cell combination and a second cell combination,

said first cell combination having an optical transmittance at a first predetermined

wavelength and a lower optical transmittance at a predetermined second wavelength, said second cell combination having a substantial light absorption at said first predetermined wavelength;

transmitting an image of at least the first and second cell combinations;

splitting and filtering said image into multiple images including at least a first filtered image in a first spectral wavelength bandwidth and a second filtered image at a second spectral bandwidth wavelength;

sensing the filtered images including the step of sensing the first filtered image with a first sensing means to provide a first electrical output representative of the first filtered image and of sensing the second filtered image with a second sensing means to provide a second electrical output representative of the second filtered image; and

providing an analysis of characteristics of the cells at a characteristic analyzer based on the electrical outputs including those from the first and second sensing operations.

4. A method of analyzing cells as claimed in claim 3 and further comprising:

providing a means for displaying any of said first image said second image and said combined image; and

coupling said display means to said means for analyzing to receive said sensed images.

5. An apparatus for the analysis of cells with at least a first cellular component and a second cellular component and with at least a first spectral material and a second spectral material, one of said first and second cellular components chemically combining with one of the first and second spectral materials to provide a first cell combination and the other of said first and second cellular components chemically combining with the other of said first and second spectral materials to provide a second cell combination,

said apparatus comprising:

a source for projecting light on said cell;

means for forming and magnifying an image of the first and second cell combinations and for communicating the image;

means for splitting and filtering the communicated image into a plurality of images including a first filtered image in a first spectral wavelength bandwidth and a second filtered image in a second spectral wavelength bandwidth,

sensing means for sensing the plurality of images including a first means for sensing the first filtered image and providing a first electrical output representative thereof and including a second means for sensing the second filtered image and providing a second electrical output representative thereof; and

means for analyzing said cell combinations and thus said cells, which analysis means is operable to receive and store the outputs of the sensing means including those from the respective first and second sensing means electrical outputs, and to combine at least said first and second images to provide an analysis therefrom, and to provide an output about at least one cell characteristic.

6. An apparatus for the analysis of cells as claimed in claim 5 wherein said means for analyzing further comprises a means for displaying said first image, said second image, said combined first and second image and said analysis thereof.